Cytokine-Glycosaminoglycan Interactions and the Role of Dermatan Sulphate-PG and TGF-β1 in the Regulation of Interferon-γ Production by Murine NK Cells

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Declaration of Authorship

I, Karishma Bavisi, hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Signed:

Date:

Abstract

Enhancing the understanding of the biological and functional role of specific cytokines in the regulation of Natural Killer (NK) cell activity is vital to cell-mediated immunity against infections and tumour development. Binding of cytokines with glycosaminoglycans (GAGs) and proteoglycans (PGs) have been known as a potential mechanism for regulating biological activity of many cytokines. In this thesis work, the binding of interleukins IL-11, IL-18 and IL-22 to heparin/HS was investigated using an ELISA approach, alongwith examination of primary sequences, 3-D protein structures and molecular docking calculations. Our results demonstrate that these interleukins do not bind to heparin/HS, which implies that the heparin-binding property should not be considered a generic characteristic for any class of cytokines. Further research focussed on examining the functional role of NK cell-surface PG, particularly dermatan sulphate (DS), in IL-12 signalled IFN-γ production using a PG synthesis inhibitor, *p*-nitrophenyl-β-xyloside. Our findings show partial inhibition of IFN-γ due to disrupted GAG/PG metabolism. Mechanistically, DS-PG interference was found independent of STAT-4 phosphorylation but suggestive of involvement essentially at the transcriptional or post-transcriptional level. In addition, our data implies that *p*-nitrophenyl-β-xyloside exerts cellular effects attenuating NK cell response to IL-12, independent of GAG-PGs. Finally, an interplay of molecular mechanisms between immunoregulatory factors, TGF-β1 and IL-12, in suppressing IFN-γ expression in mNK cells is studied. A clear evidence of partial inhibition of IL-12-induced IFN-γ production independent of NK cell proliferation is presented that does not directly involve key transcription factors of IFN-γ expression, STAT-4 and T-bet. However, promoter and early inhibition (3-6 hrs) studies suggested a possible role of TGF-β1 in chromatin remodelling of *Ifng* locus that regulates IFN-γ expression. Overall, identifying IL-heparin/HS interactions within different cytokine families and dissecting molecular events of cytokine signalling in NK cells are therapeutically important in recognising potential targets to manage cell-mediated immune responses.

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Abbreviations

APCs	Antigen Presenting Cells
Ag	Antigen
AP-1	Activator Protein -1
Arg	Arginine
ARE	AU-rich elements
AT III	Antithrombin -III
ATF-1	Activating Transcription Factor-1
Asn	Asparagine
BMPs	Bone Morphogenetic Proteins
BSA	Bovine Serum Albumin
CS	Chondroitin sulphate
CS-PG	Chondroitin sulphate- Proteoglycans
CS-A	Chondroitin -4-sulphate (type A)
CS-B	Chondroitin sulphate (type B)
CS-C	Chondroitin -6-sulphate (type C)
CD-44	Cluster of differentiation -44
CD-16	Cluster of differentiation -16
CD-64	Cluster of differentiation -64
CD-4 + T cells	Cluster of differentiation -4 positive T cells
CD-8 + T cells	Cluster of differentiation -8 positive T cells
CIA	Collagen-Induced Arthritis
CTLs	Cytotoxic T Lymphocytes
CW motif	Cardin Weintraub Motif
CMV	Cytomegalovirus
CHO cells	Chinese Hamster Ovarian cells
CREB	cAMP Response Element Binding
DC	Dendritic Cells
DS	Dermatan sulphate
DS-GAG	Dermatan sulphate - Glycosaminoglycan
DS-PG	Dermatan sulphate - Proteoglycan
DNA	Deoxy ribonucleic acid
DMSO	Dimethyl sulphoxide
ECM	Extracellular matrix
ER	Endoplasmic reticulum

ERK	Extracellular signal-Regulated Kinase
ELISA	Enzyme linked immunosorbent assay
EMSA	Electrophoretic Mobility Shift Assay
ELISA	Enzyme Linked ImmunoSorbent Assay
EAE	Experimental Allergic Encephalomyelitis
FCS	Fetal Calf Serum
FGF	Fibroblast growth factor
bFGF	basic Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor
FSC	Fat-storing cells
GAGs	Glycosaminoglycans
Gal- β	β-Galactose
D-GlcA	D-glucuronic acid
GDNF	Glial cell line -Derived Neurotrophic Factor
GlcNAc	N-acetyl glucosamine
Gal T I	Galactose transferase I
GlcUA	Glucuronic acid
GlcNAc	N-acetyl-Glucosamine
GalNAc	N-acetyl Galactosamine
GM-CSF	Granulocyte -Macrophage Colony Stimulating Factor
GPCR	G-Protein Coupled Receptor
HA	Hyaluronic acid
H	Heparin
HS	Heparan sulphate
HBD	Heparin Binding Domain
HBPs	Heparin Binding Proteins
HBS	Heparin Binding Site
HDACs	Histone Deacetylases
HGF	Hepatocyte growth factor
HS-GAGs	Heparan Sulphate -Glycosaminoglycans
IdoA	Iduronic acid
IL-11	Interleukin-11
IL-22	Interleukin-22
IL-12	Interleukin -12
IL-2	Interleukin-2
IL-4	Interleukin-4

IL-6	Interleukin-6
IL-10	Interleukin-10
IL-18	Interleukin-18
IL-12R	Interleukin-12 receptor R
IL-12R β1	Interleukin-12 receptor β1
Il-12R β2	Interleukin-12 receptor β2
IFN-γ	Interferon -gamma
IGF-I	Insulin-like growth factor I
Ig	Immunoglobulin
JAKs	Janus Kinases
KO	Knock-Out
LCMV	Lymphocytic Choriomeningitis Mammarena Virus
LM	Listeria monocytogenes
LPS	Lipopolysaccharide
Lys	Lysine
MCMV	Mouse Cytomegalovirus
MAPK	Mitogen Activated Protein Kinase
MIP-1α	Macrophage inflammatory protein -1α
MIP-1β	Macrophage inflammatory protein-1β
MHC class I and II	Major histocompatibility complex class I and class II
PAMPs	Pathogen Associated Molecular Pattern
PRRs	Pattern Recognition Receptors
PF-4	Platelet Factor -4
NFAT	Nuclear Factor of Activated T cells
NK cells	Natural Killer cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NS/NA Domains	N-Sulphated /N-acetylated domain
PGs	Proteoglycans
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDB	Protein Data Bank
RPMI-1640 media	Roswell Park Memorial Institute -1640 media
RANTES	Regulated upon Activation, Normal T-cell Expressed and secreted
RLU	Relative Light Units
RANKL	Receptor Activator of Nuclear Factor-k B ligand
L-Ser	L-serine amino acid

SCID	Severe Combined ImmunoDeficiency Syndrome
SPR	Surface Plasmon Resonance
SBE	Smad Binding Element
STAT-4	Signal transducer and activator of transcription-4
pSTAT-4	phosphorylated form of Signal transducer and activator of transcription factor -4
Th1	T-helper type 1
Th2	T-helper type 2
TNF-α	Tumour necrosis factor- α
TCR	T cell receptor
TGF- β1	Transforming growth factor-β1
Tregs	T-regulatory Cells
TSS	Transcription Start Site
TRU	T-bet Responsive unit
3'UTR	3' Untranslated region
WT	Wild Type
4-xyl β	4- β-Xylose
YY-1	Ying-Yang-1

CHAPTER 1

INTRODUCTION

1.1 The Immune System

The immune system is a diverse array of cells and their products, which collectively identifies, attacks and eradicates any infective foreign particles from the host body with minimum immunopathology. Understanding of immune system is essential to host defense and prevention of diseases. Nevertheless, improper functioning of the immune system can lead to various pathological conditions such as, infections, inflammation including autoimmune diseases, allergic disorders and cancers of the immune system (Biron & Tarrio, 2015). An overview of the immune system is described below (Chaplin, 2010). The purpose of this overview is to illustrate the interaction of different cell types in various immune mechanisms through secreted protein mediators, called Cytokines (Figure 1.1). Yet, the molecular basis of cytokine actions and immune cell activation is rather complex and less understood.

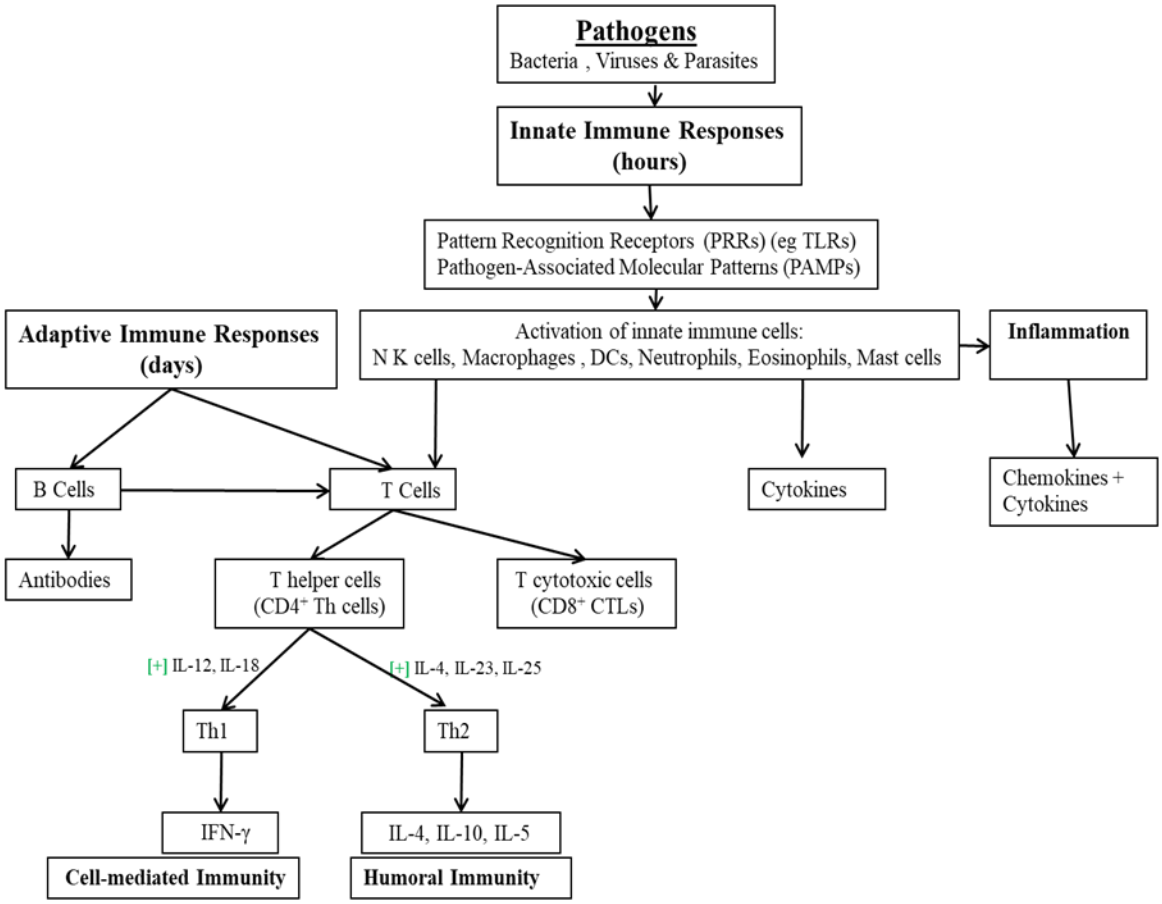


Figure 1.1: Overview of the Immune system: The figure outlines two arms of immune system, innate and adaptive immunity. In response to pathogens and their products, host body initiates innate immune response that subsequently activates components of adaptive immune responses. Activation of important cells and release of cytokines in the cascade of these immune responses are also shown. Figure modified and adapted (Goldsby *Et. al.*, 2003).

Immune cells are primarily produced in the bone marrow by a process called haemopoiesis. They circulate into bloodstream from lymphoid organs such as thymus, spleen, lymph nodes and various lymphoid tissues in the body. As illustrated in Figure 1.1, there are two types of immune responses (Alberts B., *Et. al.* 2002; Goldsby *Et. al.*, 2003; Chaplin, 2010; Hoffmann & Akira, 2013)

i) Innate immunity and

ii) Adaptive/Acquired immunity

Innate immunity is the first line of defense against infection. It is not specific for a particular pathogen, as it is triggered by molecular markers called pathogen-associated molecular patterns (PAMPs) that are conserved across a wide range of pathogens. These PAMPs on pathogens are detected by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), on innate immune cells. In response to an infection and injury, the major type of innate immune cells such as mast cells, neutrophils, basophils, monocytes and macrophages mediate an immunologic response called inflammation. A series of events takes place in this response, such as migration of innate immune cells to the site of pathogen entry, recognition of pathogen and activation of these cells by the release of cytokines, specific soluble proteins. Cytokines (detailed in Section 1.2) act as signalling molecules that regulate the function of immune cells and underlying mechanisms of defense. Moreover, cells like monocytes, macrophages, neutrophils and dendritic cells (DCs), so called phagocytic cells engulf and kill foreign objects such as bacteria by phagocytosis, an innate mechanism in the eradication of pathogens. These phagocytic cells present PAMPs as an immunologic entity on their surfaces called antigens (Ags) along with self-membrane molecules encoded by major histocompatibility complex (MHC) genes. Therefore, these innate cells are also called antigen presenting cells (APCs) and further activate adaptive immune responses.

One of the important components of innate immunity are natural killer cells (NK cells). NK cells (as detailed in Section 1.6.1) provide a non-specific, early host response due to their inherent ability to lyse tumour or pathogen-infected cells without prior sensitization. In addition to their potent cytolytic activity, NK cells secrete a range of immunoregulatory cytokines that play a significant role in eliminating pathogens. In particular, NK cells produced interferon-γ (IFN-γ), a key cytokine in the early control of invading pathogens and tumour surveillance (Ikeda *Et. al.*, 2002; Vivier *Et. al.*, 2008). Broadly, innate immunity serves two critical roles: 1) elimination or rapid

control of infectious diseases by activation of innate immune cells (phagocytes, NK cells) via secretion of cytokines that mediates cellular communications and actions; 2) leading to stimulation of a specific adaptive immune response (Roitt *Et. al.*, 1998; Mitrovic *Et. al.*, 2012b).

An adaptive or acquired immune response is characterised by specificity for a particular pathogen. Unlike innate immune cells, the adaptive response is mediated by cells called T and B lymphocytes that expresses pathogen-specific receptors on their cell surfaces. B cells recognise Ags specific to its surface immunoglobulin (Ig) receptors and present them along with MHC class II molecules to T cells. T cells use a distinct T cell-receptor (TCR) to recognize an Ag presented along with MHC I/II molecules on the surface of a self-cell such as phagocytic cells/APCs of the innate system or an altered self-cell, such as virus-infected or cancerous cell. This is how a T cell activation is different from B cells. Thus, recognition of self and non-self is another important attribute of adaptive immunity.

There are functionally two subpopulations of T lymphocytes, T cytotoxic (Tc) cells and T helper cells (Th) as distinguished by the presence of CD8 and CD4 membrane glycoproteins respectively on their cell surfaces. Tc/ $CD8^+$ T cells are activated by recognizing 'target cells', infected with a virus, or other intracellular pathogens or cancer cells in association with MHC class I molecules. On activation, the effector Tc cells called CTLs (cytotoxic T lymphocytes) exhibit cytotoxic killing of target cells. Whilst, Th/ $CD4^+$T cells are activated by Ags complexed with MHC class II molecules presented on APCs. The effector Th cells, in turn secrete various cytokines that help in the activation of other cells such as phagocytes, Tc cells and antibody-producing B cells. Th cells do not possess cytolytic activity themselves but through secretion of various cytokines, they activate other cells to destroy extracellular pathogens. Thus, Th cells regulate both innate and adaptive immune responses. Depending on the set of cytokines released by these effector Th cells, two subsets of population develop, Th1 and Th2 cells (detailed in Section 1.6.2, Figure 1.10). For example, Th1 cells are characterised by the synthesis and secretion of cytokines such as IFN-γ, IL-2, whereas, Th2 cells secrete IL-4, IL-5, IL-25, IL-13, IL-31, and IL-33 (J. Zhu & Paul, 2010). Collectively, acquired immunity contributed by T lymphocytes (Tc/ $CD8^+$ T and Th/ $CD4^+$T cells) represents cell-mediated immunity.

Interestingly, there are T cells that secrete both Th1 and Th2 cytokines, called Th0 cells. Another subset of T cells, called Th3 or T regulatory cells (Tregs), are known to downregulate immune responses by producing immunosuppressive cytokines (Crispin *Et. al.*, 2004; Wan, 2010), such as IL-10 and TGF-β that primarily antagonise lymphocyte responses (Maloy *Et. al.*, 2003). Moreover, following the clearance of infection, host responses to inflammatory reactions are suppressed by secretion of these anti-inflammatory cytokines, IL-10 and TGF-β as a mechanism in maintaining immune homeostasis (X. Ma *Et. al.*, 2015). Tregs and TGF-β1 elicit anti-inflammatory responses in control of damage caused by autoimmune diseases and regulate Th1 responses (Belkaid, 2007; Sanjabi *Et. al.*, 2017).

The research work presented in this thesis aims to enhance the fundamental understanding of NK cell activity and its role in development of Th1 immune responses, in the context of glycobiology. For this, the first part of this thesis investigates the cytokine ability in interacting with complex carbohydrates of the microenvironment such as glycosaminoglycans (GAGs)/proteoglycans (PGs) and in particular, heparin/HS. This GAG-interaction is a useful concept in understanding regulation of cytokine functions and their therapeutic application in diseases (Kufareva *Et. al.*, 2015). Of particular interest are some of the key immune cytokines, interleukin (IL)-11, IL-18, and IL-22 that have been considered for heparin/HS-binding in the current study (Chapter 3). The second part of this research seeks to investigate the underlying mechanism regulating IL-12 induced IFN-γ production in NK cells. Modulation of this NK cell response by GAGs/PGs (Chapter 4) and by immunosuppressive cytokines such as TGF-β1 (Chapter 5) is studied. This can clearly impact strategies aimed at enhancing or inhibiting NK cellular activity *in vivo* and during adoptive cell-based therapy for the benefit of immunocompromised individuals such as HIV infected or cancer patients (Morvan & Lanier, 2016).

1.2 Cytokines

As mentioned earlier in the overview of immune system, the physiologic role of cytokines is to mediate cell-to-cell communication. Cytokines are family of secreted, soluble glycoproteins, and non-immunoglobulin in nature that act non-enzymatically in nanomolar to picomolar concentrations to regulate cell function. The term ‘cytokine’ is a wider term that includes various groups of proteins such as i) interleukins- produced

by leukocytes and act on leukocytes, ii) interferons-that interfere with viral infection, iii) chemokines-chemoattractant cytokines, iv) colony-stimulating factors- support the growth of cells and v) various growth factors. They are positive or negative regulators of several immunological and non-immunological (developmental) processes such as inflammatory signalling, cell migration, differentiation, proliferation, survival and apoptosis (Goldsby *Et. al.*, 2003; Dinarello, 2007a).

Upon external stimuli, cytokines are rapidly synthesised and secreted by different cells. Unlike hormones, most cytokines are not stored in glands as preformed molecules, but have induced expression (Haddad, 2002). Hence, the expression of most cytokines is strictly regulated. Cytokine dysregulation is often manifested in many autoimmune diseases and allergic-inflammatory disorders. A clinical example most prevalent in this category is rheumatoid arthritis, an autoimmune condition which is characterised by local inflammation in the small joints of the hand and foot. At the site of inflammation, tissue damage is caused by self-reactive T cells and excessive production of pro-inflammatory cytokines such as IFN-γ (Roitt *Et. al.*, 1998; Goldsby *Et. al.*, 2003). Cytokines modulate functional activities of cells and tissues, both under normal and pathologic conditions. Therefore, with the profound role of cytokines in immune responses, they have also been considered for therapeutic intervention as vaccine adjuvants in which they act as immune stimulants and enhance the effectiveness of adoptive immunotherapies (Salgaller & Lodge, 1998; Fewkes & Mackall, 2010).

Upon secretion, cytokines bind to high-affinity receptors on target cells. They have a short half-life and most of them function in an autocrine (secretion and stimulation of the same cell) or paracrine manner (stimulation of nearby cells as target cells). Moreover, on secretion, some cytokines diffuse locally generating a concentration gradient required for their neighbouring target cells to act in a concentration-dependent manner. Therefore, cytokine activity is mainly localised around the source of secretion. Except in immune reactions against systemic infections, in which, macrophages and other innate immune cells in the circulation secrete large amounts of cytokines that may be active beyond the site of secretion called endocrine mode of action. A clinical scenario that parallels such a condition is called ‘septic shock syndrome’ that is characterised by high levels of cytokines, TNF-α, IL-12 and IFN-γ in the blood, setting a systemic inflammation due to the response of the immune system to infection. A detectable level of cytokines in the systemic circulation is a disease biomarker (St

Ledger *Et. al.*, 2009). Consequently, in pathologic states, cytokines circulate in an endocrine manner, however physiologically, these soluble factors stimulate immune responses in a paracrine mode. Moreover, the localised delivery of cytokines in a therapeutic application has been reviewed as an effective alternative to the systemic route of administration in vaccine delivery system (Salem *Et. al.*, 2010). Therefore, this raises a question as to how these soluble and diffusible cytokines are retained locally within the tissue sites.

Studies conducted almost three decades ago, by Dexter and Spooncer, showed that haemopoiesis in bone marrow cells co-cultured with stromal cells could be sustained for several months*in-vitro* , without exogenously added cytokines. Moreover, the cytokines were not detected in the culture supernatant. This led them to propose that haemopoietic cytokines being synthesised must be retained within the stromal cell layer, after secretion (Dexter & Spooncer, 1987). At the time of this finding, it was known that marrow-derived stromal extracellular matrix (ECM) contained special polysaccharides called glycosaminoglycans (GAGs) (Dexter, 1987). In parallel, with the work presented by Dexter and Spooncer, Gordan et al provided evidence that among GAGs, particularly, heparan sulphate (HS) on the stromal cell surfaces were able to bind haemopoietic cytokines like IL-3 and GM-CSF (Gordon *Et. al.*, 1987). Moreover, HS-bound cytokines were biologically active to stimulate stem-cell proliferation and differentiation (Gordon *Et. al.*, 1987; Roberts *Et. al.*, 1988). Thus, this was suggested as the mechanism behind the *in-vitro*, long-term survival and maintenance of haemopoiesis in bone marrow cultures. The outcome of this early work highlighted that cytokines bind to GAGs which are present on the cell surface or ECM. Moreover, cytokine binding to GAGs results in selective retention of these factors close to the site of their secretion and paracrine signaling. Such localization of cytokine activity also contributed to the formation of compartmental niches defining the marrow microenvironment where the binding facilitated the regulation of hematopoietic stem-cell growth and differentiation. Thus, it was understood that the role of cytokines in cell-regulation and immune responses is modulated by the GAG components of the ECM or microenvironment.

Since then, a number of key immune proteins like cytokines have been shown to bind to GAGs and the number is steadily increasing. Consequently, GAG-binding has emerged as one of the regulatory mechanisms that involves a combined requirement

for diffusible (cytokine) and non-diffusible (matrix) signals to localise the responses of cells to a cytokine that is secreted (Ruoslahti & Yamaguchi, 1991; Roy & Kornberg, 2015). Such a mechanism has been the basis for the explanation of cytokine and leukocyte functions in various immune processes, like leukocyte trafficking, microbial adhesion and infection, regulation of inflammation at the sites of infection or antigen exposure (Tanaka *Et. al.*, 1993; Taylor & Gallo, 2006), and most importantly in angiogenesis and tumour development (Chiodelli *Et. al.*, 2015; Peysselon & Ricard-Blum, 2014). Thus, the implications of cytokine-GAG interactions have been recognized in both innate and adaptive immunity (Davis & Parish, 2013; Coombe & Parish, 2015; Kang *Et. al.*, 2018). In the recent years, understanding the interaction of immunologically significant cytokines with GAGs at the molecular level and the functional significance of such interactions is a growing field of interest. This would allow for therapeutic manipulation of cytokine actions in the treatment of immunological diseases. In the subsequent sections, GAGs/PGs are introduced with respect to their structure, function and biosynthesis that contribute towards binding to proteins, importantly, cytokines.

1.3 Glycosaminoglycans (GAGs) and Proteoglycans (PGs)

GAGs/PGs are one of the most diverse forms of biological macromolecules. They are present on the surface of almost all cell types, in the ECM and in the circulation (Iozzo & Schaefer, 2015). Structurally, GAGs are linear polysaccharide chains covalently attached to serine residues of a core protein to form complex macromolecules called proteoglycans (PGs) (Gallagher, 2015) (Figure 1.2). Alternatively, the protein core of a PG is a scaffold onto which GAG chains are attached. Moreover, PGs contain a relatively higher polysaccharide content, compared to protein found in glycoproteins (reviewed in Prydz, 2015; Pomin & Mulloy, 2018).

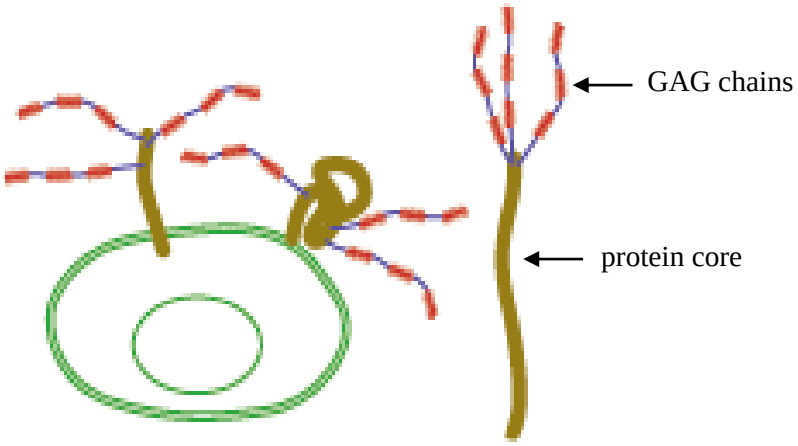


Figure 1.2: Proteoglycans: Proteoglycans (PGs) consists of a protein core (brown in color) to which glycosaminoglycan (GAG) chains are covalently attached (red and blue color). This figure illustrates (A) cell-surface PGs, attached to membranes or spanning the bi-layered cell membrane (green) and (B) Extracellular matrix (ECM) PGs, which are usually secreted or may shed from cell-surface PGs. Figure is modified and adapted from (Lindahl & Kjellen, 2013)

1.3.1 Structure and Functions

As mentioned earlier, GAG chains are unbranched, polysaccharide chains of variable size and composition. They are made up of specific, repeating disaccharide units of sugar acids such as uronic acid, either D-glucuronic acid (GlcUA) or L-iduronic acid (IdoA) that alternates with a sugar amine, either N-acetyl-glucosamine (GlcNAc) or N-acetyl galactosamine (GalNAc) (Gallagher, 2015; Xu & Esko, 2014). Moreover, one or both of the sugar units contain sulphate residues; therefore, GAGs are highly sulphated and strongly negatively-charged macromolecules (Prydz & Dalen, 2000; Pomin & Mulloy, 2018).

Based on the particular disaccharide repeat unit, GAGs can be divided into 4 subgroups (Prydz & Dalen, 2000; Mulloy & Rider, 2006), as shown in Figure 1.3.

A) Heparin (H) and Heparan Sulphate (HS),
B) Keratan Sulphate (KS),
C) Chondroitin Sulphates (CS; chondroitin sulphate-A [CS-A], chondroitin sulphate-B [CS-B] or Dermatan Sulphate (DS) and chondroitin sulphate-C [CS-C])

D) Hyaluronic Acid (HA). HA is an exception, as it is synthesised as a free GAG i.e. without a core protein and lacks sulphate.

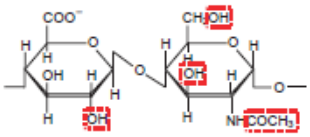
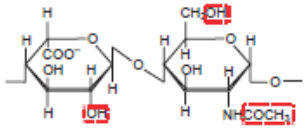
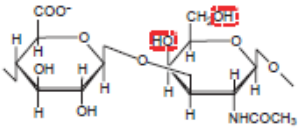
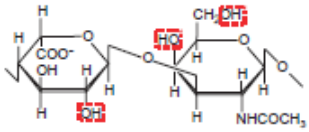
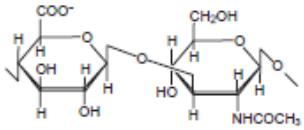
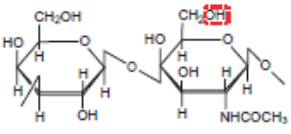
GAG (Composition)	Sugar acid	Sugar amine-N-acetylated	Disaccharide structure
Heparin/Heparan Suphate	D-glucuronic acid (GlcA) and Iduronic acid (IdoA)	D-glucosamine (GlcNAc)	 GlcA β(1→4) GlcNAc α((1→4)  IdoA α(1→4) GlcNAc α(1→4)
Chondroitin Sulphate	D-glucuronic acid (GlcA)	D-galactosamine (GalNAc)	 GlcA β(1→3) GalNAc β(1→4)
Dermatan Sulphate	D-glucuronic acid (GlcA) and Iduronic acid (IdoA)	D-galactosamine (GalNAc)	 IdoA β(1→3) GalNAc β(1→4)
Hyaluronic acid	D-glucuronic acid (GlcA)	D-glucosamine (GlcNAc)	 GlcA β(1→3) GlcNAc β(1→4)
Keratan Sulphate	Galactose	D-glucosamine (GlcNAc)	 Gal β(1→4) GlcNAc β(1→3)

Figure 1.3: Disaccharide structures and composition of different types of GAGs: GAGs consist of repeating disaccharide units, each unit with an uronic acid and an amino sugar, except keratan sulphate (sugar- highlighted in yellow). Possible sites of sulphation in the disaccharide unit are denoted in red. Figure is modified and adapted from (Prydz & Dalen, 2000).

GAGs and hence PGs have a prominent role as structural components. For example, PGs containing HA in ECM gives cartilage a gel-like property that resists deformation in joints. This hydrating property of HA has been used in making specific HA-based formulations in regenerative medicines (Highley *Et. al.*, 2016). Additionally, PGs/GAGs exhibit a range of functions depending on their composition of sugar residues and/or location (Powell *Et. al.*, 2004). A CS-PG called Versican, found in many ECMs are ligands to receptors, TLR2 (toll-like receptor 2) and TLR6 of innate immune cells (W. Wang *Et. al.*, 2009). Moreover, GAGs/PGs associated with cell membranes are known to function as cell-adhesion molecules, anchoring cells to the ECM fibres and involved in cell signalling (A. C. Rapraeger, 2000). This is well-illustrated by Syndecan, a cell-surface PG containing HS/CS-GAG chains on its external domain binds to fibrous proteins (collagen and fibronectin) present in the interstitial matrix surrounding the basal lamina (Afratis *Et. al.*, 2017). Perlecan, a cell-surface HS-PG is known to bind and accumulate various growth factors in the ECM (Whiltelock *Et. al.*, 2008). GAGs like heparin, are also found segregated into intracellular granules in secretory cells such as mast cells. These GAGs are released during cell degranulation at the sites of inflammation or tissue injury (Mulloy *Et. al.*, 2017). Thus, the biological function of PGs is diverse, ranging from structural and mechanical support in tissues to the interaction with various growth factors and proteins.

The interaction of PGs with proteins is prominently via the GAG components which, in turn, results in modulation of protein function. Proteins that bind to GAGs belong to various classes including cell adhesion molecules, morphogenetic proteins, plasma proteins, enzyme-inhibitors, proteins involved in lipoprotein uptake, hormones, and cytokines like chemokines and interleukins (Esko & Linhardt, 2009; Ori *Et. al.*, 2011; Pomin, 2015). A number of scenarios have been illustrated in Figure 1.4 that shows binding to GAGs is an important mechanism underlying the biological outcomes of many of these proteins. Classically, one of the best characterised interaction between GAGs and proteins is the activation of a serpin protease inhibitor, antithrombin-III (AT III) and heparin/HS which leads to inhibition of blood coagulation cascade (Choay *Et. al.*, 1983; Petitou *Et. al.*, 2003; Mosier *Et. al.*, 2012). GAG-protein interactions are a means of localising concentrations of growth factors in the ECM or at cell surfaces at the sites of their production (J. T. Gallagher & Turnbull, 1992b; Johnson *Et. al.*, 2005). Moreover, binding to GAGs prevents degradation by proteases of the ECM (Saksela

Et. al., 1988; Lortat-Jacob & Grimaud, 1991c; Clarke *Et. al.*, 1995). This further allows the bound and preserved protein to be presented to its specific cell-surface receptor initiating signal transduction. Such an interesting interaction has been demonstrated in the case of fibroblast growth factor (FGF), which binds tightly to the HS chains in the extracellular domains of PGs (recent reviews Gallagher, 2015; Meneghetti *Et. al.*, 2015). This HS-bound FGF is presented to its high affinity polypeptide receptor, FGFR on the cell membrane to initiate signal transduction in cell growth and proliferation (Yayon *Et. al.*, 1991; Itoh *Et. al.*, 2011). The role of heparin was proposed to induce dimerisation of FGF molecules thereby facilitating dimerisation of respective receptors, FGFRs and hence their activation (Pellegrini *Et. al.*, 2000; Schlessinger *Et. al.*, 2000; Brown *Et. al.*, 2013). Because of this, heparin/HS were referred to as co-receptors for FGF signalling. Such early work on GAG-bound FGFs has led to the hypothesis that GAG-binding prevents diffusion of growth factors and retention close to the site of secretion supporting paracrine or juxtacrine mode of action (J. T. Gallagher & Turnbull, 1992b; R. Xu *Et. al.*, 2012).

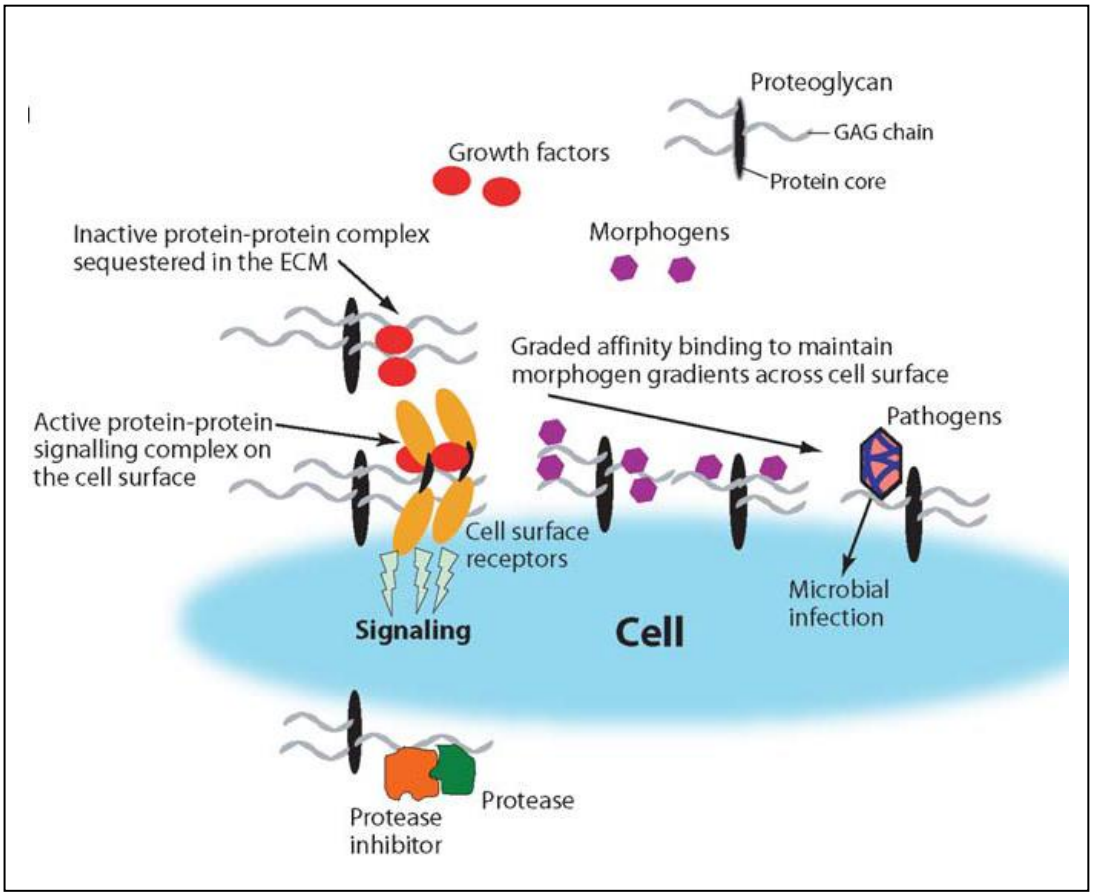


Figure 1.4: GAG-protein interactions: The figure shows the interaction of GAG chains of PGs with proteins that result in sequestration of proteins close to the site of secretion, presentation of GAG-bound protein to cognate membrane-receptors in signal transduction, oligomerisation leading to concentration gradient in attracting migration of leukocytes, protection from proteases in ECM or circulation. Each interaction depicts a specific influence of GAGs on biological functions of bound proteins. Figure from (Sasisekharan *Et. al.*,2006).

Evidence to localisation of activity has also been observed during inflammatory reactions by chemokines (Kufareva *Et. al.*, 2015; Proudfoot *Et. al.*, 2017). Binding of chemokines to heparin/HS have shown to form concentration gradients that importantly provides directional cues to circulating neutrophils (Johnson *Et. al.*, 2005; Rajarathnam *Et. al.*, 2018). Therefore, GAG-binding property directs to a better understanding of either sequestration or presentation of cytokine activity. Further, it is well-exemplified by a heparin-binding cytokine, IFN-γ (Lortat-Jacob & Grimaud, 1991a; Lortat-Jacob & Grimaud, 1991c; Saesen *Et. al.*, 2013) that binding to GAGs confers additional stability to cytokines. IFN-γ is an important cytokine in early host defense and is vital to both innate and adaptive immune responses (Schoenborn & Wilson, 2007; Larkin *Et. al.*, 2013). Studies have shown that free IFN-γ in circulation is cleaved along its length at carboxyl-terminal rendering the cytokine inactive. Whereas heparin-bound IFN-γ has shown to be protected from extensive proteolytic processing by circulating proteases that increases the half-life of IFN-γ in the circulation from 1.1 to 99 minutes, thus increasing its biological efficacy (Lortat-Jacob *Et. al.*, 1996a). Moreover, IFN-γ binding to heparin/HS modulates the receptor interaction and hence bioactivities of this key cytokine in tissues (Yard *Et. al.*, 1998, Fernandez-Botran *Et. al.*, 1999) Collectively, the role of GAGs in affecting physiological events by binding to cytokines is many-fold; they induce oligomerisation, regulate ligand-receptor interaction i.e. affect biological activity by competing with cytokine membrane-receptors. Further, they modify carboxyl-terminal processing, thereby increasing the stability and bio-availability of cytokines. The nature and functional consequences of individual GAG-cytokine interaction are different and have distinctive outcomes. Since, cytokines are small proteins with a short half-life in blood, GAG-induced cytokine activity and stability play a significant role when a cytokine is evaluated in a pharmacological or clinical application (Adage *Et. al.*, 2012; Gerlza *Et. al.*, 2015; Weiss *Et. al.*, 2017). Thus, binding of a cytokine to GAGs like heparin/HS have major consequences on cytokine function, and that functional influence is different among cytokines.

1.3.2 Biosynthesis of GAGs and PGs

The molecular basis to the binding ability of GAGs to many different classes of proteins is the structural complexity and diversity which arises from their biosynthesis (Esko & Lindahl, 2001; Gandhi & Mancera, 2008). Therefore, it is important to understand this process of GAG synthesis. The overview of the process is as described below and depicted in Figure 1.5 (Prydz & Dalen, 2000; Esko & Selleck, 2002; Mulloy & Rider, 2006). The biosynthetic machinery to produce GAGs and PGs is largely located in the Golgi apparatus (Prydz & Dalen, 2000; Esko & Lindahl, 2001). PG synthesis begins with the sequential addition of series of monosaccharide units onto a core protein structure to form a long unbranched polysaccharide chain with specific disaccharide repeats. The biosynthetic process is fast; complete synthesis of an entire GAG chain is achieved in 1-3 min (Esko, 1991). This process takes place in three stages namely chain initiation, chain elongation and modification.

Chain initiation involves transfer of the first sugar, D-xylose (Xyl) from UDP-xylose to a serine or threonine residue of the protein core by the enzyme, xylosyl transferase. To this xylosylated protein core, two galactose (Gal) residues and a glucuronic acid (GlcUA) unit are then added sequentially to form the tetrasaccharide core linkage region (GlcUA-Gal-Gal-Xyl). This tetrasaccharide linkage region is the starting point for polysaccharide chain elongation. Chain elongation then starts with the addition of fifth sugar to a tetrasaccharide linkage region. Importantly, if the fifth sugar residue is *N*-acetylglucosamine (GlcNAc), it commits the process towards heparin/ HS chain synthesis, whereas, for CS and DS chains, *N*-acetlygalactoseamine (GalNAc) is added. The protein core dictates the type of GAG synthesized and attached (Gallagher, 2015). This is followed by alternate addition of GlcUA residue to form a disaccharide repeat. Thus, the elongation process results in a long, non-sulphated, unbranched polysaccharide chain (Esko & Selleck, 2002).

Whilst the GAG chain is still undergoing polymerization, it is modified by a series of sequential modification reactions. These modifications add significant charge density and structural complexity to the polymer chain (Kjellen & Lindahl, 1991; J. T. Gallagher *Et. al.*, 1992a). For Heparin/HS, which are the types of GAGs with highest structural diversity (Kreuger & Kjellen, 2012), the *N*-acetyl glucosamine (GlcNAc) in the polymerised chain undergoes modifications with *N*-acetyl groups being replaced

with sulphate groups. One of the four isoforms of enzyme *N*-acetylglucosamine *N*-deacetylase/*N*-sulphotransferase (NDST) could catalyse this reaction and has been recognised as a single protein with dual functionality. *N*-deacetylation/*N*-sulphation are concomitant processes *in vivo* and, therefore, free amino groups on glucosamines are rare (Aikawa *Et. al.*, 2001). *N*-sulphation is a prerequisite for all subsequent modifications such as *O*-sulphation (U. Lindahl *Et. al.*, 1986) or C5 epimerization of GlcUA to IdoA. This is because *N*-sulphate groups are necessary for substrate recognition by the respective enzymes. *N*-sulphation is carefully regulated during synthesis, resulting in fine structural characteristics of heparin/HS chains that can often be distinguished in different cells (J. T. Gallagher & Walker, 1985).

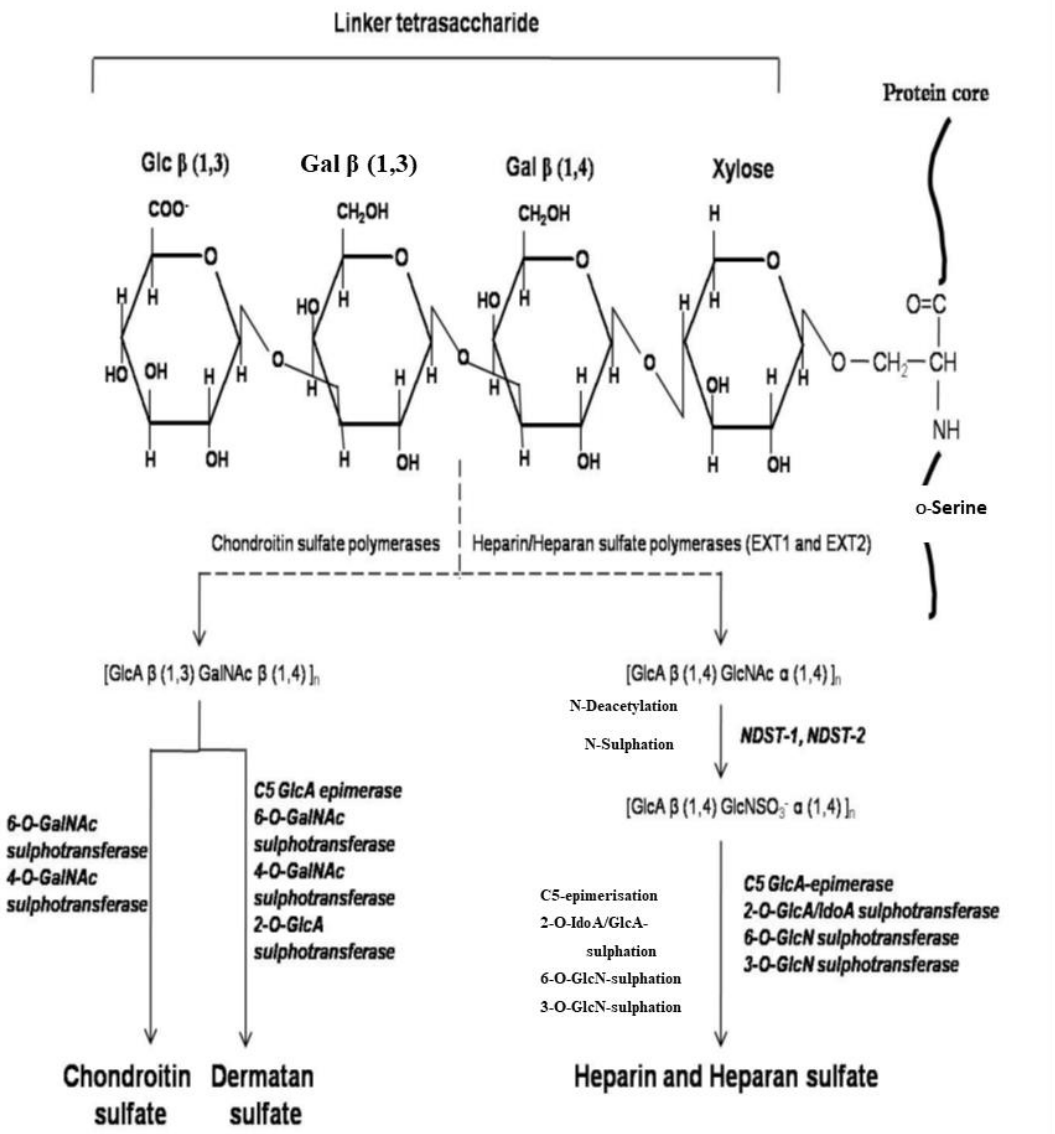


Figure 1.5: Steps in the biosynthesis of GAGs: Stepwise synthesis of different types of GAGs – heparin/HS, CS, and DS form from a common tetrasaccharide linkage region present on core protein. Various post-modification reactions catalysed by biosynthetic enzymes such as NDST, 2OST (2-O-sulphotransferase), 6OST (6-O-sulphotransferase), C5 epimerase which modifies glucuronic acid (GlcUA) to iduronic acid (IdoA) at C-5 have also been indicated as described in the text. Figure modified and adapted from (Prydz & Dalen, 2000).

Following the *N*-sulphation, the glucosamine can additionally be *O*-sulphated at C-6 and occasionally at C-3 positions by *O*-sulphotranferase enzymes or at both C-3 and C-6 or no sulphates. There are 6 isoforms of 3-*O*-sulphotranferase (3-OST) expressed at different levels in different tissues to form tissue-specific saccharide sequences (J. Liu *Et. al.*, 1999). Therefore, based on the sulphation patterns, glucosamine in

heparin/HS chain can be found in different states, such as *N*-acetylated/*N*-sulphated/free amino with *N*- and 3 or 6 *O*-sulpho groups. Moreover, the glucuronic acid (GlcUA) in heparin /HS either remains without modification in some disaccharide units, or undergoes epimerisation at C5 to IdoA, which can further be 2-*O* sulphated. Thus, GlcUA can exist in four variable states; unmodified, epimerised, *N*-sulphated, and *O*-sulphated. Combining the possibilities of variable states for both *N*-acetyl glucosamine and glucuronic acid, the number of modification reactions could result in up to 48 combinations per disaccharide unit of heparin/HS-GAGs (Sugahara & Kitagawa, 2002). This indicates that the potential for variations in even short oligosaccharide sequences is enormous in GAGs and this variation exceeds extraordinarily compared to other biological macromolecules like DNA (combination of 4 different units) or proteins (20 different units). However, out of all theoretically possible disaccharides, only around half of them have actually been found in GAGs, possibly due to limited substrate specificity offered by the enzymes involved in GAG biosynthesis (reviewed in (Esko & Selleck, 2002; Mulloy & Rider, 2006).

An important aspect to understand about the mechanism of GAG biosynthesis is that it is not template-driven, only a proportion of polysaccharide chains are modified by enzymes (and their tissue-specific isoforms) leaving considerable scope for sequence diversity (Gallagher, J.T. and Lyon, M., 1989). Moreover, the sequential modifications of the polysaccharide chain catalysed by multiple biosynthetic enzymes do not undergo completion. Therefore, GAG biosynthesis is regulated by the expression and activity of these enzymes that, in turn, dictates structural heterogeneity in GAG chains.

In the case of HA, it is not subjected to any post-modification reactions. KS and CS, however, undergo less modification than heparin/HS. Indeed, the heparin/ HS class has greater sequence variation through modifications compared to other GAGs; hence they have higher anionic charge density (Kreuger & Kjellen, 2012). Therefore, most GAG-binding proteins interact with high affinity to heparin/HS. DS is derived from CS with the incorporation of IdoA (Sugahara *Et. al.*, 2003). The presence of IdoA adds to the conformational flexibility in the modified regions of heparin/HS and DS chains that are most involved in binding with proteins. For this reason, many proteins including cytokines, bind to heparin/HS and DS over other GAGs. Indeed, the sulphation patterns which results in overall charge density and variations in GAG structures at the molecular level generates diverse interactions with proteins that translate into

physiological or pathological changes in the microenvironment (Tanaka *Et. al.*, 1993; Taylor & Gallo, 2006; Lindahl & Kjellen, 2013).

1.3.3 Heparin and Heparan Sulphate (HS)

As understood from the above sections that among GAGs, heparin and heparin sulphate (HS) are highly sulphated and are of particular importance for their almost universal existence. Among these GAGs, heparin is a highly sulphated variant of HS that is exclusively found in mast cell granules and is released on degranulation, during inflammation. HS is, however, widely distributed and is found on the cell surface of all mammalian cells and in the extracellular matrix as part of PGs (Bishop *Et. al.*, 2007). Both are widely used GAGs due to their anticoagulant property (Capila & Linhardt, 2002). Heparin is easy to obtain, more homogenous, and commercially available in bulk quantity as an anticoagulant. Therefore, for simplicity, rather than HS, heparin is employed for most experimental and theoretical studies, including the research work presented in this thesis.

Although, heparin and HS share the same repertoire of saccharide residues – glucuronic acid (GlcA) 1-4 linked to N-acetyl glucosamine (GlcNAc), there is an important difference in the structures of heparin and HS (Gandhi & Mancera, 2008; Gallagher, 2015). Compared to heparin, HS polysaccharide chains are distinctly organised into domains as illustrated in Figure 1.6, containing different types and densities of modifications, including sulphation, that are spaced apart along the chain (J. Turnbull *Et. al.*, 2001; Rabenstein, 2002). Short regions along the polysaccharide chain that are highly modified and sulphated are called NS domains and this region resembles heparin chains. NS domains are found separated by longer regions with fewer modifications and low sulphation, mainly consisting of GlcA-GlcNAc repeats called *N*-acetylated or NA domains. The NA and NS domains are separated by 'mixed domains' containing, *N*-acetylated and *N*-or *O*-sulphated GlcNAc as well as GlcA or IdoA or IdoA2S (J. T. Gallagher, 2006). The NS and mixed domains are termed hypervariable regions that result in different functional characteristics for HS from different cell types (Coombe & Kett, 2005). Consequently, HS shows overall greater structural variation than heparin (Bernfield *Et. al.*, 1999). Such structural pattern arises because of modification reactions that occur in clusters during biosynthesis, leaving regions either devoid of sulphation or low sulphation along the chain. This

characteristic structure of HS also differentiates it from other GAGs, which have more uniform structure and sulphation throughout the polysaccharide chain (Maccarana *Et. al.*, 1996).

NS domains are typically of 3-8 disaccharide units in length with distinct clusters of sulphate groups particularly *N*- or 3-, or 6-*O*- sulphated positions in close proximity with or without epimerisation of glucuronic acid (GlcA) to iduronic acid (IdoA) (Lyon & Gallagher, 1998b) as shown in Figure 1.6. IdoA may be sulphated at 2-*O*-position adding to overall charge density to the chain (J. E. Turnbull & Gallagher, 1991). Introduction of IdoA residues into NS-domains is a crucial modification that provides flexibility to the chain due to its variable conformational states and additionally influences the spatial rearrangement of sulphate, carboxylate, and hydroxyl groups attached to it and to adjacent sugars (Raman *Et. al.*, 2003; Raman *Et. al.*, 2005). The nature and pattern of modifications varies from one source of HS to another resulting in different compositions of NS domains, depending on cell or tissue type (U. Lindahl *Et. al.*, 1998; Shi & Zaia, 2009). Therefore, the variation in NS-domain composition could imply another level of regulation in binding and modulating the activity of local cytokines, growth factors, and various other protein ligands by GAGs. Even though NA-domains are not directly involved in protein interaction, they act as spacers between two NS-domains of protein binding sites. Spacing of the NS domain has been observed as an important factor in the ability of heparin/HS chains to interact with proteins (Mobli *Et. al.*, 2008). For example, interaction between HS and multimeric platelet factor-4 (PF4) involves typical S-A-S domains, where S represents the NS domain and A is the NA domain. Thus, two NS regions, each as a binding site, were found on opposite sides of the PF4 tetramer separated by unsulphated NA region (Stringer & Gallagher, 1997b).

Although HS features alternating NA-domains and NS-domains, the levels of sulphation in NS-domains match the levels seen in heparin that interact with proteins (U. Lindahl *Et. al.*, 1998; Mulloy & Forster, 2000; Gallagher, 2015). In comparison to HS, heparin exhibits shorter stretches of NA domains and contains a high ratio of IdoA/GlcA and more of *N*-sulphated glucosamine. Thus, heparin chains are characterised by long stretches of tri-sulphated dissacharide units, IdoA2SGlcNSO$_3$6S (Yamada *Et. al.*, 1998). Therefore, both GAGs differ in their degree of sulphation, with heparin displaying an average disaccharide containing 2.7 sulphate groups while HS,

although biosynthetically related to heparin, contains only one sulphate or less per disaccharide, depending on the source (Hileman *Et. al.*, 1998; Gandhi & Mancera, 2008).

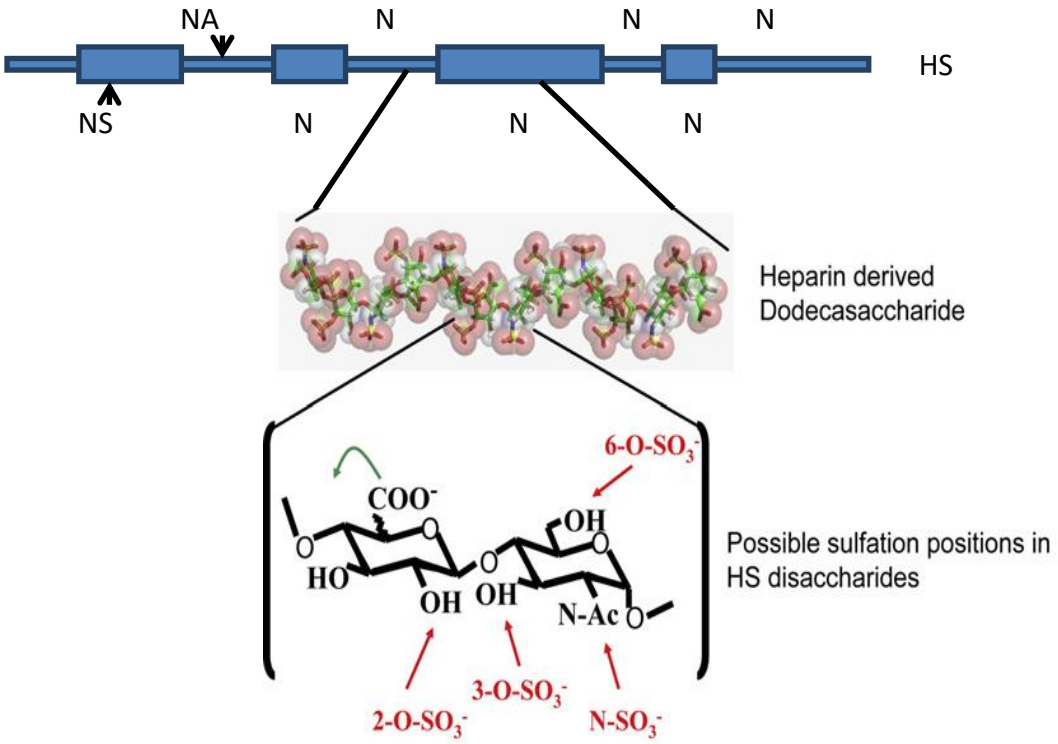


Figure 1.6: Strutural Organisation in Heparin/HS: Heparin/HS are complex polysaccharide chain with disaccharide repeat units of glucosamine and glucoronic acid. As shown heparin/HS chains are organized into domains of high sulphation (NS domains) are separated by low sulphation regions dominated by N-acetylated glucosamines (NA regions). Structural heterogeneity in their chains arises from varying number and position of sulphation that results in domain structure. Modified and adapted (Laguri *Et. al.*, 2008).

Additionally, the solution structure of heparin is well-defined and determined by Mulloy *Et. al.* using NMR and molecular modelling techniques (Mulloy *Et. al.*, 1993; Mulloy & Forster, 2000). According to this (Figure 1.7), heparin chains exists in helical conformation with glucosamine (GlcN) and glucuronic acid (GlcA) moieties in fixed conformations as well as rigid glycosidic linkages. Whilst, the IdoA residues are flexible to fluctuate between two conformations, $^{1}C_{4}$ chair and $^{2}S_{0}$ skew-boat. These two conformations of IdoA are in equilibrium in solution (Das *Et. al.*, 2001) and they orient the bulky carboxylate and 2-*O*-sulphate groups of IdoA in rather different

positions on the helical axis. Therefore, such relatively rigid and bulky structure of heparin can have significant impact on protein binding.

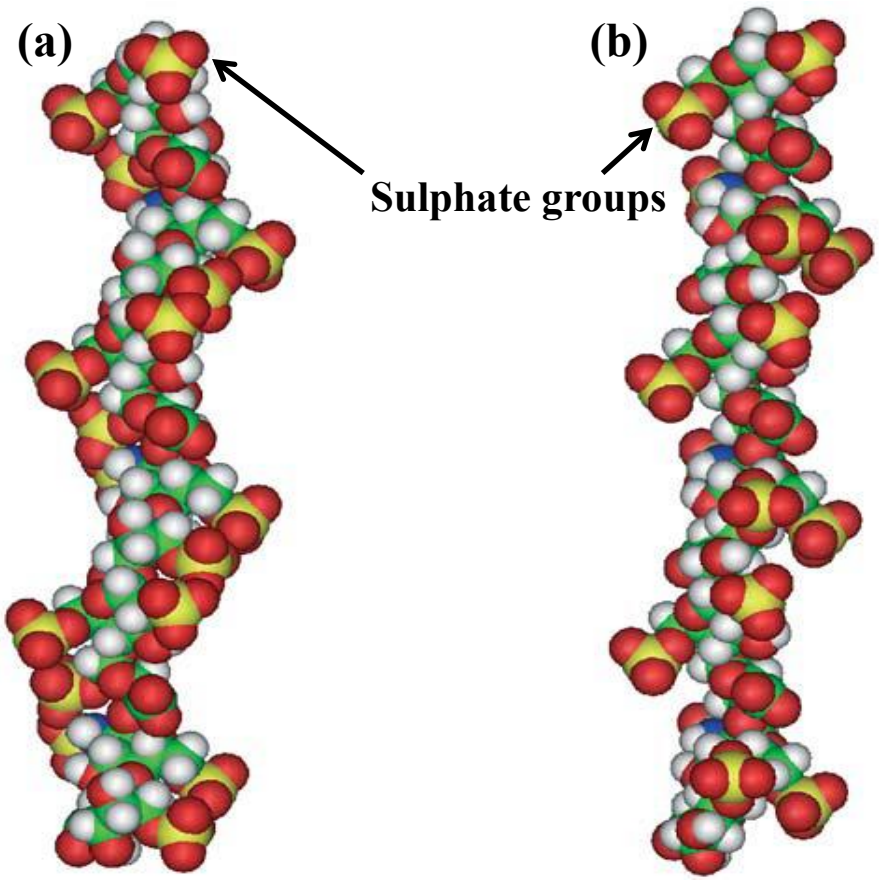


Figure 1.7: Solution structure of Heparin: Solution structure of heparin determined by NMR (Mulloy *Et. al.*, 1993) is represented in a space-filled style and was first presented in Mulloy & Forster 2000. The iduronates in these heparin hexasaccharides can be seen in (a) $^{1}C_{4}$ chair and (b) $^{2}S_{0}$ skew-boat conformations. As seen, heparin adopts a helical conformation and its sulphate groups (sulphur atoms coloured yellow and oxygen atoms colored red) align on the opposite faces of the helical axis. Modified and adapted from (Coombe & Kett, 2005).

According to the structure described for heparin (Mulloy *Et. al.*, 1993), it is tri-sulphated disaccharide repeat. The arrangement of sulphate groups on heparin chains is such that a group of three sulphate ions are clustered on one side of the chain and alternates with a similar cluster of the next disaccharide unit on the other side of the chain (Mulloy & Forster, 2000), as shown in Figure 1.7. In one of the proposed models of heparin-mediated FGF oligomerisation (Pellegrini *Et. al.*, 2000), two FGF-1 molecules were on the opposite faces of a single heparin chain implicating the functional importance of sulphate clusters on either side of the helical conformation (Mulloy *Et. al.*, 2005). The solution structure of heparin has been deposited in the

Protein data bank (PDB) under the code 1HPN (Mulloy *Et. al.*, 1993) and has been used in the current study of molecular docking to predict the location of heparin binding sites in selected cytokines (Chapter 3, Section 3.2.2). On the other hand, there is very little information on the three-dimensional structure of HS. Apparently, the NS domains of HS may exist in helical conformation of heparin (Khan *Et. al.*, 2013; Gallagher, 2015).

1.4 Cytokine - Heparin/HS Binding

As described earlier, that for many cytokines binding to heparin/HS in the ECM or on cell-surfaces largely influences their biological activity. This led several research groups including Dr. Rider's group to determine the binding of cytokines to a range of acidic polysaccharides representing heparin/HS and other GAGs (Ramsden & Rider, 1992). Subsequently, our laboratory (Dr. Rider's group) investigated and characterised many immunologically and developmentally significant GAG-binding cytokines such as IL-1α, IL-1β (Ramsden & Rider, 1992), IL-2 (Najjam *Et. al.*, 1997a; Najjam *Et. al.*, 1997b), IL-12 (Hasan *Et. al.*, 1999), IL-6 (Mummery & Rider, 2000), GDNF (Rickard *Et. al.*, 2003), Artemin, Neurturin (Alfano *Et. al.*, 2007), β-cellulin (Mummery *Et. al.*, 2007) and BMP-7 (McClarence, 2011). However, what was interesting to our group and many other laboratories with similar studies in the field (Lortat-Jacob *Et. al.*, 1990; Tanaka *Et. al.*, 1993), was the observation that cytokines exhibited selective binding affinity amongst GAGs. For example, IL-2 was shown to bind heparin, highly sulphated HS and fucoidan, a sulphated polysaccharide from brown algae but did not bind to CS, DS and HS with low sulphation levels (Ramsden & Rider, 1992; Najjam *Et. al.*, 1997a). Similarly, IL-7 bound to heparin/HS and fucoidan, but displayed no affinity for CSA, KS or HA (Clarke *Et. al.*, 1995; Zhang *Et. al.*, 2012). Such observed variations in the binding profile of each cytokine led to an understanding of the nature and specificity in the GAG-cytokine interactions. Apparently, there is some degree of structural specificity contributed by each binding partner such as saccharide sequences and distinct sulphate groups on GAG chains that are recognised by regions or domains composed of basic amino acids on the cytokine (protein) structure. Even today, there is a considerable amount of research understanding the nature of protein-heparin/HS interactions (Xu & Esko, 2014). Subsequent sections describe the basis of cytokine-heparin interactions, that is the nature and specificity of the interactions and finally

highlights the characteristics of heparin-binding sites/domains (HBS/HBD) on cytokines.

1.4.1 Nature of Cytokine-Heparin/HS interaction

As learnt from the structure of heparin/HS in the previous section (Section 1.3.3), the most prominent and common feature of these polysaccharide chains is the density of sulphate and carboxylate groups which contributes to the overall negative charge. Therefore, these sugar polymers are by nature highly acidic which suggests an electrostatic affinity towards proteins rich in basic amino acids. The basic residues that mainly contribute towards heparin/HS binding are arginine and lysine (Caldwell *Et. al.*, 1996; Fromm *Et. al.*, 1997; Hileman *Et. al.*, 1998). Arginine as a basic residue has shown to offer 2.5 times stronger binding compared to lysine on a basis of relative affinity for heparin. This is due to additional, non-ionic interactions that support binding affinity such as hydrogen bonds formed by the guanidinio group of Arg with sulphate groups on heparin/HS (Fromm *Et. al.*, 1995). Consequently, these cytokine-heparin/HS interactions are largely dominated by simple ionic interactions between negatively charged sulphate and carboxylate groups on heparin/HS and the side chains of positively charged basic amino acids of cytokines (Fromm *Et. al.*, 1997; Capila & Linhardt, 2002). In addition to basic residues, the presence of other polar amino-acids with small side chains such as glycine (G), serine (S), or uncharged sidechains like asparagine (N) and glutamine (Q) within or around the heparin-binding domains were shown to stabilise and further support the interaction with heparin. This was due to the formation of additional non-ionic interactions such as hydrogen bonds with the anionic groups on heparin/HS that further contributed to the specificity and affinity of binding (Hileman *Et. al.*, 1998; Gandhi & Mancera, 2008; Sarkar & Desai, 2015).

Besides the ionic interactions, a significant contribution from the non-ionic bonds in the interaction between the proteins and heparin/HS have been noted. Indeed, a structural study of AT III in complex with a heparin pentasaccharide showed that ionic interactions formed by basic residues contributed only 40% of the binding energy. Whereas, the remaining 60% of the binding energy was contributed by non-ionic interactions, such as van der Waals forces, hydrogen bonds and hydrophobic interactions of basic residue side-chains with the carbohydrate backbone of the heparin/HS chains (Bae *Et. al.*, 1994; Jairajpuri *Et. al.*, 2003). Similarly, crystal

structures of the complex between a heparin hexasaccharide and FGF-2 have demonstrated a significant number of these non-ionic forces that have added to the existing specificity and increased the binding affinity between heparin and FGF-2 (Faham *Et. al.*, 1996; Adage *Et. al.*, 2012). Therefore, even if non-ionic interactions play a significant part, the ionic interactions are critical in heparin binding and heparin-induced protein activity (Hileman *Et. al.*, 1998; Gandhi & Mancera, 2008).

1.4.2 Specificity of the Interactions

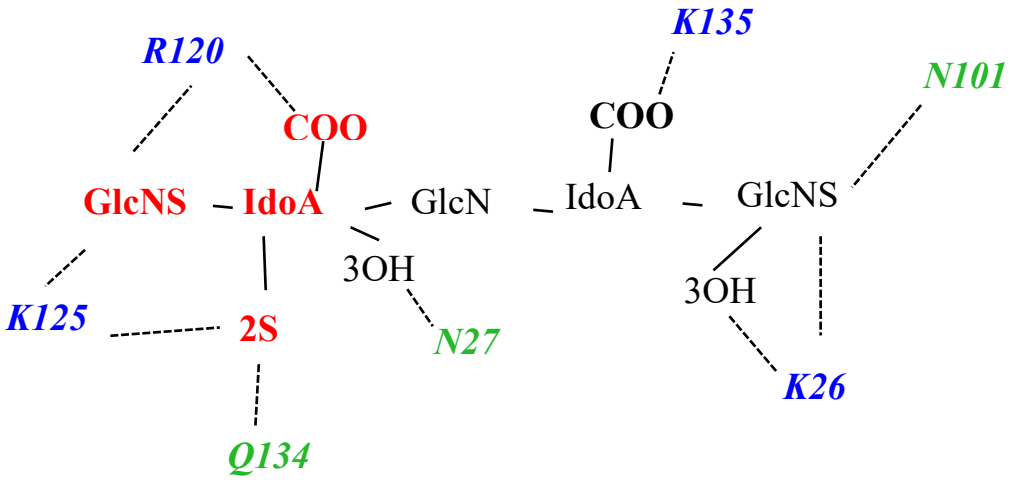
While it is clear that the principal interactions are between the basic residues of cytokines and highly sulphated acidic polysaccharides, heparin/HS are ionic interactions, it is not merely based on overall charge complementation. Specificity is predominantly within ionic interactions between particular sulphate and carboxylate groups along the heparin chain that imparts negative charges and the arrangement of specific basic residues (Lys and Arg) on protein surfaces that confer positive charges (Hileman *Et. al.*, 1998, Kreuger *Et. al.*, 2006). Moreover, non-ionic interactions add to specificity and also increases affinity in interactions (Adage *Et. al.*, 2012). Therefore, a fully sulphated heparin chain increases the overall anionic charge density but the involvement of selective sulpho-groups within an oligosaccharide sequence brings specificity to the heparin-cytokine binding (Kreuger *Et. al.*, 2006; Varki *Et. al.*, 2009; Kayitmazer *Et. al.*, 2010). For example, the interaction between AT-III and heparin occurs with high affinity through a defined pentasaccharide sequence. The key feature of the pentasaccharide sequence is a rare 3-*O*-sulphated glucosamine (Choay *Et. al.*, 1983). However, the presence of an *N*-sulphated and 6-*O*-sulphated glucosamine at the centre of the oligosaccharide also influences the affinity (Jin *Et. al.*, 1997; Petitou *Et. al.*, 2003; Mosier *Et. al.*, 2012). Similarly, a trisaccharide sequence (GlcNS,6S-IdoA,2S-GlcNS,6S) containing *N*-sulphated and 6-*O* sulphated glucosamines intervened by 2-*O*-sulphated iduronic acid (Maccarana *Et. al.*, 1993) has been identified in high affinity interactions of heparin with most FGFs. Within this sequence the presence of a 6-*O* sulpho group on the glucosamine residues are required for direct interaction with FGF-1 proteins (Kreuger *Et. al.*, 2001) but are not involved in FGF-2 binding with heparin (A. Walker *Et. al.*, 1994; Rusnati *Et. al.*, 1994). Rather, FGF-2 binds to the same *N*-sulphated sequence with 2- *O*- sulphated IdoA as the key moiety in the interaction (Faham *Et. al.*, 1996). This shows different levels of specificity within members of the same family in the interaction with heparin/HS (Faham *Et. al.*, 1998;

Raman *Et. al.*, 2005; R. Xu *Et. al.*, 2012). However, it is now widely accepted in the field that the association of distinct sulphate groups in the interaction with proteins offers the higher degree of specificity rather than the saccharide sequences per se, in protein recognition by GAGs (as reviewed in Mizumoto *Et. al.*, 2013; Chiodelli *Et. al.*, 2015; Meneghetti *Et. al.*, 2015).

From a proteomic side, the specific basic residues involved in the interaction could be from the same part of the linear sequence of the primary structure, representing a consensus 'sequence motif' as found in number of heparin-binding proteins (Cardin & Weintraub, 1989). Binding of domains containing linear sequence of basic amino acids to heparin/HS chains has been suggested to induce local conformational changes in protein that may attract additional basic residues in close contact to anionic groups leading to higher affinity and specificity (Cardin & Weintraub, 1989; Raman *Et. al.*, 2005). Alternatively, a positively charged cluster may be a 'conformational characteristic/site' that is formed on higher structural folding of protein. Such a conformational fold brings in together the basic residues distant in sequence and lead to protein-specific binding (Capila & Linhardt, 2002). Thus, the heparin/HS-binding domain (HBD) on proteins [as detailed in next section 1.4.3] is an important site with a specific arrangement of basic residues that recognises and facilitates best-fit binding with specific anionic-groups distributed on heparin chains (Hileman *Et. al.*, 1998; Raman *Et. al.*, 2005; Kreuger *Et. al.*, 2006; D. Xu & Esko, 2014; Meneghetti *Et. al.*, 2015). As seen in Figure 1.8A below, the heparin/HS binding sequence in FGF-2 is shown with the key basic residues highlighted. Further in Figure 1.8B are the key contacts in the FGF-2-heparin complex. The highlighted section in Figure 1.8B shows specific interactions with Lys 125 and Arg 120 as critical residues of the domain. Although major interactions are ionic, Asn and Gln residues offer support by forming H-bonds (Adage *Et. al.*, 2012; Gallagher, 2015).

^{117}A-L-**K**-**R**-T-G-N-Y-**K**^{125}-L-G-S-**K**-T-G-P-**Q**-**K**^{135}

(A) FGF-2: peptide sequence of FGF-2 involved in heparin/HS binding



(B) Key basic residues in FGF-2 in contact with heparin/HS sequence

Figure 1.8: Molecular interaction between FGF-2 and heparin: A) Heparin-binding sequence in FGF-2 highlights key basic residues (blue) and polar, uncharged residues (green) involved in heparin interactions **B**) Schematic representation of molecular contacts in FGF-2-heparin complex. Lys (K125) and Arg (R120), key basic residues in heparin-binding domain of FGF-2 forms ionic bonds with specific anionic groups (carboxylate, sulphate ions) on saccharide sequence of heparin (red). In addition to basic residues, Asn (N27, N101), Gln (Q134) support the interaction with hydrogen bonds. Figure adapted from (Gallagher, 2015).

1.4.3 Identification of Heparin/HS-Binding Sites/Domains (HBSs/HBDs) on Cytokines

Studies on various heparin/HS-protein complexes have provided useful insight into the features of heparin/HS-binding sites or domains in proteins as recently reviewed in (Billings & Pacifici, 2015; Gallagher, 2015). In general, HBSs are interactive sites on the surface of proteins, comprising a cluster or group of basic amino acids (mainly Arg/Lys)forming a positively-charged surface for binding with negatively-charged, sulphate and carboxylate groups of heparin/HS chains. Besides containing a high number of basic residues, it is the arrangement of these residues on the protein surface and the spacing with non-basic amino acids that constitutes a heparin-binding site or domain (HBS/HBD) (Caldwell *Et. al.*, 1996; Fromm *Et. al.*, 1997; Hileman *Et. al.*,

1998; Gandhi & Mancera, 2012). A HBS is recognised as the linear array of basic residues in the primary sequence of proteins called 'sequence-motifs'. Screening of the primary sequence is a useful starting point to reveal basic amino acids close in sequence that could be possibly involved in the interaction with GAG chains.

Cardin and Weintraub (Cardin & Weintraub, 1989) were the first to screen a number of heparin-binding proteins to identify common structural elements within HBDs. They identified the presence of two consensus 'sequence-motifs' in the primary sequence of more than 20 heparin-binding proteins. These were XBBXBX and XBBBXXBX, where B is any basic amino acid and X is any hydropathic amino acid [such as hydrophobic and polar-uncharged amino acids, Asn (N), Ser (S), Gly (G), Ala (A), Leu (L), Ile (I), Tyr (Y)] (Hileman *Et. al.*, 1998; Gandhi & Mancera, 2008). Since then, these motifs in the primary sequence were commonly called Cardin and Weintraub motif (CW motif). According to this theory, the basic residues in the CW motif line-up along one side on the surface of a secondary structure fold (either a β-strand or α-helix) while the hydrophobic residues point towards the protein core. Such orientation enables the basic residues to interact with the sulphate groups on heparin chains. The Cardin and Weintraub motif was used as one of the most common search parameters among the few other proposed motifs for the initial screening of HBSs/HBDs on proteins (Fromm *Et. al.*, 1997; Hileman *Et. al.*, 1998). This has also been useful to predict if a protein would possibly interact with heparin/HS.

Nevertheless, the universal acceptance of the outcome from this theoretical approach has been challenged by the subsequent advancements in the field, such as site-directed mutagenesis. For example, a study on GDNF demonstrated that an N-terminal deletion mutant of GDNF which lacks the CW motif, BXBB, still binds to heparin (Alfano *Et. al.*, 2007). Notably, cytokines like IL-4 (Lortat-Jacob *Et. al.*, 1997), IL-8 (Webb *Et. al.*, 1993; Kuschert *Et. al.*, 1998), and GM-CSF (Roberts *Et. al.*, 1988) bind to heparin even though they lack CW motifs. Additionally, with the increasing number of heparin-binding proteins being discovered, only a few residues from the consensus sequence-motif were found to be involved in heparin binding. Although these findings suggest that the presence of a Cardin-Weintraub motif in a protein is not essential for heparin binding, the presence of one could support the possibility of such an interaction. Therefore, the screening and analysis of primary sequence of proteins is particularly

useful in predicting and correlating these consensus motifs to heparin-binding domains in proteins, whose three-dimensional structure is not known.

Clusters of basic residues that are easily evident from the primary sequence of protein are called continuous heparin-binding sites. Whereas, those clusters that are formed by the distant basic residues brought together in a three-dimensional protein folding constitute discontinuous heparin-binding sites (Mulloy & Rider, 2006), also referred as conformational domains/sites (Gallagher, 2015). Therefore, a HBS does not always comprise of basic amino acids in a linear sequence, rather basic amino acids from different secondary structural elements of a protein can come close together on higher conformational folding (R. Xu *Et. al.*, 2012; Capila & Linhardt, 2002). For example, FGF-2 contains a HBS comprised of basic residues K26, N27, R81, K119, R120, T121, Q123, K125, K129, Q134, and K135 (Figure 1.8). These residues exhibit close spatial proximity but are not close in primary sequence (L. D. Thompson *Et. al.*, 1994; R. Xu *Et. al.*, 2012). Multiple studies of heparin-protein complexes using structural analytical techniques, such as X-ray crystallography (Mulloy and Linhardt 2001), NMR (Kuschert *Et. al.*, 1998), and molecular modelling (Lortat-Jacob *Et. al.*, 2002; Mulloy & Forster, 2008) also support this concept.

A typical heparin-binding domain/site consists of 4-6 basic residues in a single cluster within the linear arrangement of amino acids, or on the surface of a higher order of a structurally folded protein (Hileman *Et. al.*, 1998). Moreover, heparin being a helical structure with relatively rigid and bulky groups section 1.3.3) (Mulloy *Et. al.*, 1993), means heparin chains cannot access clefts and folds within protein structures. Consequently, the cluster of basic amino acids must be exposed prominently onto the protein surfaces for interaction with heparin chains. Such a cluster on proteins typically forms surface structures such as shallow groove or cavity with a specific arrangement of surface basic residues (Rullo & Nitz, 2010). Additionally, analysis of a number of co-crystal structures of protein-heparin complexes have shown that the basic residues comprising a HBD were suitably spaced and oriented to match the pattern of sulphate groups along the heparin/HS chain (Raman *Et. al.*, 2005; Sasisekharan *Et. al.*, 2006). Supporting this, heparin was shown to induce α-helical structures in polylysine peptides such that the periodicity of sulphate clusters on one side of the heparin chain approximately matched the periodic turns on the α-helix, allowing ionic interactions with the basic charges of lysine residues (Mulloy *Et. al.*, 1996). Thus, the distribution

of basic residue clusters and their accessibility to GAG-heparin/HS chains on protein surfaces are important factors in determining heparin-binding ability and sites on proteins.

Overall, with the knowledge and understanding of specific structural features on heparin/HS for binding to a particular cytokine, novel therapeutic agents, such as modified oligosaccharides or heparin mimetics as competitive inhibitors are generated for medical intervention in the cytokine network of inflammatory diseases (Casu *Et. al.*, 2010; Mohamed & Coombe, 2017) including cancer (Peysselon & Ricard-Blum, 2014; Chiodelli *Et. al.*, 2015; Weiss *Et. al.*, 2017). Synthetic GAG analogs such as commercially available short carbohydrate chains or molecules charged with acidic groups that lacked the anti-coagulant activity of heparin were shown to exhibit anti-inflammatory effects *in vitro* as well as *in vivo* in disease-specific models of inflammation (Severin *Et. al.*, 2012). Thus, various strategies exploit the potential of heparin/HS as anti-inflammatory agents with its reduced or eliminated anti-thrombotic property (Rek *Et. al.*, 2009). A non-sulphated K5 polysaccharide derived from *E. Coli* has shown anti-viral activity (Rusnati *Et. al.*, 2009). Heparin/HS mimetics such as PI-88, PG-545 to block cancer growth metastasis are currently in clinical trials (Liu *Et. al.*, 2014).

Identification of heparin-binding sites on cytokines can be used in synthesizing anti-inflammatory, mutant cytokines that are devoid of any affinity for GAGs. Such chemokine variants of MIP-1β and RANTES have been used in *in vivo* studies. These variants failed to exhibit their biological functions of recruiting cells compared to the wild type (WT) counterparts (Martin *Et. al.*, 2001), suggesting an important role of heparin/HS in chemokine function directing cell migration. Heparin/HS binding is required to maintain local concentrations of chemokines for attracting leukocytes and to induce oligomerisation for chemotactic activity (Proudfoot *Et. al.*, 2003). When a mutant to chemokine-RANTES, ^{44}AANA^{47}-RANTES was injected intraperitoneally, it was effective in antagonising cell recruitment compared to its WT in three murine models of inflammation such as Experimental Allergic Encephalomyelitis (EAE) and liver fibrosis (Johnson *Et. al.*, 2004). Thus, cytokine-heparin interactions provide insight into potential advantages in the development of novel cytokine mutants for therapeutic intervention. Recent focus in the field has been in engineering chemokine-based decoy proteins with enhanced GAG-binding ability and reduced or knocked-out

G-Protein Coupled Receptor (GPCR)-binding affinity (Adage *Et. al.*, 2012; Gerlza *Et. al.*, 2015). As a result, these variants in preclinical models were shown to displace WT chemokines in binding GAGs but are unable to activate leukocytes. Overall, this reduces cellular trafficking in an inflammatory response and hence controls inflammation-associated tissue damage. Currently this approach is in clinical development as CellJammer® technology (Adage *Et. al.*, 2012; Adage *Et. al.*, 2015). Such chemokine variants have shown potential in preventing infection like CCL-5 based decoy proteins are engineered as potential HIV entry inhibitors (Kufareva *Et. al.*, 2015) and CCL-2 decoy proteins in blocking tumour metastasis (Roblek *Et. al.*, 2016).

1.5 Cytokine Candidates for Heparin/HS Binding Studies

Cytokines belonging to a particular family or subfamily show remarkable structure-function relationship (Rozwarski *Et. al.*, 1994). Even though, members of a particular family show low amino acid sequence homology, they exhibit similar topology in their three-dimensional or tertiary structures (Bazan, 1990). As part of functional similarity, family members bind to homologous receptors (Bazan, 1990; Sprang & Bazan, 1993). It is interesting to raise a question here, whether a heparin-binding property is common among cytokines that share structural relationship with their family members known to bind heparin/HS. For example, the chemokines, a large family of chemoattractant cytokines, contain about 50 members within four sub-families and all act via GPCR (G-protein-coupled transmembrane receptors) for signaling (Rossi & Zlotnik, 2000; Lortat-Jacob *Et. al.*, 2002; Bachelerie *Et. al.*, 2014). These members have relatively low sequence similarity, but they possess similar tertiary structures with the same monomeric folds (Schwarz & Wells, 1999; Lortat-Jacob *Et. al.*, 2002). Their receptor binding shows cross-reactivity, in that a single receptor can be recognized by many chemokines and vice versa (Rossi & Zlotnik, 2000; Proudfoot, 2002). Interestingly, the majority of chemokines bind to heparin/HS *in vitro* (Hoogewerf *Et. al.*, 1997) and *in vivo* (Proudfoot *Et. al.*, 2003) and these GAGs are required for the biological function of chemokines *in vivo* (Proudfoot, 2006; Kufareva *Et. al.*, 2015; Proudfoot *Et. al.*, 2017).

Similarly, a quarter of members of the TGF (Transforming Growth Factor)-β superfamily are known to bind heparin/HS (Mulloy & Rider, 2006). GFLs (GDNF family ligands) and BMPs (bone morphogenetic proteins) are cytokine subfamilies

belonging to the TGF-β superfamily. These cytokines exhibit a characteristic, conserved cysteine-knot structure that results in shared tertiary structural fold in them. (C. C. Rider, 2006; Rider & Mulloy, 2010). Most members of the GFL subfamily, like GDNF (glial-cell-line-derived neurotrophic factor), artemin and neurturin are sequence homologues and possess heparin-binding activity (Rickard *Et. al.*, 2003; Alfano *Et. al.*, 2007). In this context, heparin/HS binding is a widespread property among BMPs (BMP-2, -4, -7) and their protein antagonists such as follistatin, gremlin, noggin (Rider & Mulloy, 2010; McClarence, 2011; Gandhi & Mancera, 2012). Therefore, one can observe that many, if not all, members of a cytokine family/subfamily possess heparin-binding property.

This brings us to the first goal of this thesis (chapter 3) that investigates whether the three selected interleukins, IL-11, IL-18 and IL-22, bind to heparin/HS. These interleukins are thus far to be tested for their heparin-binding property and they share structural relationship with the respective heparin-binding members of the IL family. This will also provide an important insight about the extent of heparin-binding activity within the IL family. As described below, each of these three interleukins are key candidates for study because they possess basic residue clusters or sequences which are characteristic of heparin-binding domains. Besides, these interleukins play a central role in various immune responses and have significant therapeutic potential in diseases; therefore, it becomes highly relevant to investigate their ability to bind heparin/HS. Since binding to heparin/HS modulates the biological activities of cytokines, even non-binding characteristics would draw attention to their physiological role. Thus, this investigation will provide a valuable insight into cytokine-heparin/HS interactions in the activation and regulation of immune responses. Further, as these interleukins share cytokine-family relationships, findings from this study would highlight differences in the functional implications of heparin binding within the same cytokine family. With regards to therapeutic application, as most recombinant interleukins have been limited by toxicity associated with systemic administration, investigating heparin-binding properties or lack thereof, may enable to the reduction of undesirable side-effects by supporting a localised mode of cytokine delivery and paracrine activity. Subsequent subsections introduce these interleukins, IL-11, IL-18 and IL-22 in more depth with respect to their biological importance and structural features, as compared to their heparin-binding counterparts from the respective family.

1.5.1 Interleukin-11

Interleukin-11 (IL-11) is a stromal cell-derived, haemopoietic cytokine (Paul *Et. al.*, 1990). It has strong lymphopoietic activity and acts alone or synergistically with other growth factors such as IL-3, GM-CSF to regulate multiple events in the development of myeloid and lymphoid cell lineages (Du & Williams, 1997). Importantly, IL-11 stimulates platelet production and therefore, rHuIL-11 is commercially available as 'oprelvekin' from Neumega ® for therapeutic use in the prevention of chemotherapy-induced or bone marrow failure-associated thrombocytopenia (Demetri, 2000). Moreover, clinical studies have successfully demonstrated IL-11 as an effective agent for improving the recovery of platelet levels and reducing the need for platelet transfusion after myelo-suppressive cancer chemotherapy (Bhatia *Et. al.*, 2007). In addition to the role of IL-11 in cancer care, this soluble factor has gained attention as a systemic biomarker for various inflammation-inducing pathological stimuli (Negahdaripour *Et. al.*, 2016). Indeed, recent evidence showed elevated expression of IL-11 with its central role in linking chronic inflammation of gastric epithelia to gastrointestinal cancers in humans (Putoczki & Ernst, 2010; Putoczki *Et. al.*, 2013). Further findings of the emerging role of IL-11 in breast cancer and tumour progression has very recently been reviewed in Johnstone *Et. al.*, (2015). Other non-haematopoietic functions of IL-11 include osteopoietic activity which regulates osteoclast development and bone metabolism (Sims *Et. al.*, 2005). Biologically, IL-11 downregulates IL-12 production by macrophages (Leng & Elias, 1997), and thereby modulates NK cell and T cell mediated inflammatory cytokine production and Th1 immune responses, suggesting a possible therapeutic role in bone marrow transplantation (Hill *Et. al.*, 1998; Bozza *Et. al.*, 2001). Knowing these biological and clinical implications of IL-11 (Negahdaripour *Et. al.*, 2016), it is potentially an interesting candidate to assess for it's heparin-binding properties. The binding of IL-11 with ECM or cell-surface HS-GAGs may be an underlying mechanism controlling its important bioactivities.

Structurally, IL-11 is a member of a family of long-chain cytokines within a superfamily of four α-helical cytokines (Nichola N. A. 1994). Six cytokines including IL-11, IL-6, leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotropin-1 (CT-1) use a common receptor-subunit, glycoprotein (gp)-130 for signal transduction. Therefore, this group is called the gp-130 receptor family of cytokines, or IL-6-type cytokines (Hawley, 1994; Heinrich *Et. al.*, 2003). These

cytokines share biological activities due to overlapping signal transduction pathway that recruit and subsequently lead to homo/heterodimerisation of gp-130 (Kishimoto *Et. al.*, 1994; Dahmen *Et. al.*, 1998). Many of the biological effects described above for IL-11overlap with those of IL-6 (Musashi *Et. al.*, 1991; Barton *Et. al.*, 2000; Matadeen *Et. al.*, 2007; Negahdaripour *Et. al.*, 2016), which is a known heparin-binding cytokine (Mummery & Rider, 2000). Moreover, a secreted human IL-11 is a 178-amino acid polypeptide. Notably, the primary sequence of IL-11 contains 20 basic residues including a cluster containing four basic residues within an eight-residue sequence. Indeed, the arrangement of basic residues within this sequence closely resembles a classic Cardin-Weintraub motif. Collectively, this shows a good probability for interaction of IL-11 with polyanionic heparin/HS chains. Therefore, this led us to hypothesise that heparin-binding may be a common property in the gp-130 receptor-cytokine family. To test this hypothesis, IL-11 was selected as one of the candidates from the respective family to study possible binding to heparin.

1.5.2 Interleukin-18

Interleukin-18 (IL-18) is a pro-inflammatory cytokine produced by monocytes and macrophages against microbial infections (Gracie *Et. al.*, 2003). IL-18 was formerly called IFN-γ inducing factor (IGIF) and is associated with severe inflammatory conditions like sepsis and autoimmune diseases [as recently reviewed in (Dinarello *Et. al.*, 2013)]. Therefore, IL-18 has shown to be a valid clinical target to combat the pathological consequences of sepsis via IFN-γ (Dinarello & Fantuzzi, 2003; Dinarello, 2007b). Biologically, IL-18 alone or synergistically with IL-12, functions to activate T and NK cells to produce IFN-γ, stimulates Fas-mediated NK cell cytotoxicity, and regulates the development of Th1 cells (Okamura *Et. al.*, 1995; Dinarello, 1999). Administration of recombinant IL-18 has been reported to have antitumour activity in murine models (Micallef *Et. al.*, 1997). IL-18 has gained recent attention as a key cytokine in patients with metabolic diseases like type-1 (Harms *Et. al.*, 2015) and type-2 diabetes mellitus (Troseid *Et. al.*, 2010). Interestingly, high circulating levels of IL-18 in metabolic syndrome has been suggested to be central to atherosclerotic lesions and other inflammatory pathogenic effects (as reviewed in Troseid *Et. al.*, 2010). Given the functional importance of IL-18, determining a heparin-binding activity in IL-18 may help in understanding the basis for the *in vivo* effects of this cytokine in the circulation.

Structurally, IL-18 belongs to the IL-1-like β-trefoil cytokine family based on similarities at several levels (Dinarello, 2002; Kato *Et. al.*, 2003; Kato *Et. al.*, 2014). With the IL-1 family member, IL-1β, a heparin-binding cytokine (Ramsden & Rider, 1992), IL-18 shares the cleavage enzyme site for caspase-1 or IL-1β converting enzyme (ICE) to process proIL-18 into a mature and biologically active form (Bazan *Et. al.*, 1996). Receptors for IL-18 belong to the IL-1 receptor family and connect signal transduction pathways by recruiting a similar downstream component, NF-kB (Parnet *Et. al.*, 1996). At the three-dimensional structural level, IL-18 exhibits a β-trefoil structural fold which comprises of twelve β-strands arranged to form three anti-parallel four-stranded β-sheets, set in a triangular fashion to form a pyramid–like structure (Kato *Et. al.*, 2003). This β-trefoil structure is a signature feature to those of IL-1 and FGF family members (Murzin *Et. al.*, 1992). It has been observed that the topological positions of primary receptor sites for IL-1β and FGF-2 are super-imposable (Kato *Et. al.*, 2003). Hence, IL-18 also extends similarity in its three-dimensional structural scaffold with FGF family members. IL-18 is a protein enriched in basic amino acids with a cluster near the carboxyl-terminal, encompassing β strands β10-β11 and their connecting loop. This region, between β strands, β10-β12 near the carboxyl-terminus is of particular significance, as it conforms to a heparin-binding domain in FGFs (Faham *Et. al.*, 1998; Ornitz & Itoh, 2001a). The FGF family presently contains 22 members and each member exhibits a β-trefoil structural fold (Ornitz & Itoh, 2001a; Asada *Et. al.*, 2009). Despite differences in their amino acid sequences, these FGF members exhibit an identifiable, although varying, degree of heparin-binding activity (Raman *Et. al.*, 2003; Asada *Et. al.*, 2009). On a structural basis, it raises the possibility that IL-18 also shares this property with FGF proteins, a family of well-characterised heparin-binding cytokines. In this context, GAGs like heparin/HS may restrict the diffusion of IL-18 when secreted, favoring a paracrine rather than endocrine effect and may further facilitate receptor recognition or activation, as observed for FGF cytokines.

1.5.3 Interleukin-22

Interleukin-22 (IL-22) is an inflammatory cytokine secreted by IL-9 stimulated mast cells and activated T and NK cells (Dumoutier *Et. al.*, 2000a; Witte *Et. al.*, 2010). An interesting aspect of this cytokine is that it is secreted by immune cells, but the activity is mainly targeted to non-immune cells, especially hepatocytes, keratinocytes and epithelial cells to induce pro-inflammatory activities such as the production of acute

phase proteins (Wolk *Et. al.*, 2004; Wolk & Sabat, 2006a; Zenewicz & Flavell, 2011). Clinical studies suggest the role of IL-22 is critical in mucosal immunity of the lung and gut (Aujla *Et. al.*, 2008; Zheng *Et. al.*, 2008). Although IL-22 has important physiological effects in local tissue damage and repair through proliferation, survival and regeneration of epithelial cells (Sabat *Et. al.*, 2014), these same effects of the cytokine were recently implicated in pathological inflammation such as cancer (Lim & Savan, 2014) and autoimmune diseases (Pan *Et. al.*, 2013). Elevated levels of IL-22 were detected in psoriasis, a human autoimmune disease of the skin (Lowes *Et. al.*, 2007; Ma *Et. al.*, 2008). IL-22 induces hyper-proliferation and induction of inflammatory proteins in skin keratinocytes resulting in characteristic psoriatic lesions (Boniface *Et. al.*, 2005) and recruitment of immune cells to the sites of psoriatic inflamed tissue (Wolk *Et. al.*, 2006b). As heparin/HS is a key regulator of most biological processes including cell proliferation, inflammation (D. Xu & Esko, 2014; Esko *Et. al.*, 2009), it will be interesting to know whether IL-22 binds to these ubiquitous GAGs and whether this association is required to exert its biological effects.

Given the influence of IL-22 in host defense and disease conditions, it is an attractive target for clinical development (Dudakov *Et. al.*, 2015). Moreover, continued interest in IL-22 has set this cytokine as a well-studied example among the members of the IL-10 family of cytokines. Structurally, IL-22 is α-helical cytokine member of the interferon-like/IL-10 family, along with IFN-α, -β, -γ, IL-10 and IL-19 as other members (Pestka *Et. al.*, 2004; Trivella *Et. al.*, 2010). IL-22 shares 22% amino acid sequence identity, and conservation of the first three of four cysteine residues with its structural homolog, IL-10 (Dumoutier *Et. al.*, 2000b). Despite low sequence homology, IL-22 shares a strong secondary structural homology with IL-10 that contains six α-helices in each monomeric domain of a dimer (Nagem *Et. al.*, 2002; T. Xu *Et. al.*, 2005). Moreover, most of the conserved residues between IL-22 and IL-10 are located in the carboxyl-terminal domain of the protein. This domain has been found to be critical for IL-10 activity that suggests IL-22 and IL-10 may share common or related biological activities (Nagem *Et. al.*, 2002). IL-22 exhibits overlapping but distinct signalling pathways to IL-10 (Moore *Et. al.*, 2001). The biological activity of IL-22 is initiated by binding to heterodimeric cell surface receptor subunits, IL-22R1 and IL-10R2, a shared component of IL-10 signalling. However, IL-22R1 defines the action of IL-22 signal transduction in cells (Bleicher *Et. al.*, 2008; Jones *Et. al.*, 2008a). Both IL-22 receptors belong to the interferon class II receptor family and shows sequence

homology to the receptors of IFN-γ, which functions as an inflammatory cytokine (Langer *Et. al.*, 2004). Interestingly, IL-22 also shares significant structural similarities to IFN-γ, a helical cytokine, like IL-10 (Nagem *Et. al.*, 2002). Both IL-10 (Salek-Ardakani *Et. al.*, 2000; Kunze *Et. al.*, 2014; Kunze *Et. al.*, 2016; Gehrcke & Pisabarro, 2015) and IFN-γ (Lortat-Jacob *Et. al.*, 1991b; Saesen *Et. al.*, 2013) are known heparin-binding cytokines. Additionally, the carboxyl-terminus of human IL-22 features a cluster of four basic residues within eight residue sequence which resembles the heparin-binding, carboxyl-terminal peptide of IL-10 and IFN-γ. This increases the likelihood of IL-22 binding to heparin/HS. Thus, a structure-function relationship with these two heparin-binding cytokines makes IL-22 an appropriate candidate for the current study.

1.6 Regulation of Interleukin (IL)-12 Induced IFN-γ production in NK cells

As mentioned at the outset of this chapter (Section 1.1), the second aspect of this thesis deals with the regulation of IL-12-signalled IFN-γ production in NK cells. NK cells are one of the critical players of innate immunity and IL-12 plays a key role in the initial activation of NK cells, thereby, in the production of innate IFN-γ (Zwirner *Et. al.*, 2010; Marcais *Et. al.*, 2013). NK cell-derived IFN-γ is important in the cross-talk between innate and adaptive immunity (Vivier *Et. al.*, 2008). For instance, during viral infections such as CMV, DCs secrete chemokines and cytokines such as IL-12, which in turn activates NK cells to produce IFN-γ. Chemokines secreted were shown to recruit peripheral NK cells to the T-cell rich zones of lymph nodes to provide an early source of IFN-γ, essential for priming antigen-specific, $CD4^+$ T cells and Th1 cell-polarisation (Martin-Fontecha *Et. al.*, 2004). Supporting this, more recent studies clearly highlight that IFN-γ production by NK cells regulates susceptibility to infections (Fodil *Et. al.*, 2014; Freeman & Hill, 2015) and tumour development (Morvan & Lanier, 2016). This thesis, therefore, addresses the IFN-γ producing pathway in NK cells in response to IL-12 stimuli. Although such pro-inflammatory functions of IL-12 in IFN-γ production are well characterised and essential for innate immunity (Hamza *Et. al.*, 2010; Vignali & Kuchroo, 2012), if left uncontrolled, the physiological response may lead to induction of autoimmunity or inflammatory shock due to overproduction of IFN-γ (S. K. Lee *Et. al.*, 2012; Lees, 2015). Given the critical role of IFN-γ in mediating NK cell functions, regulation in expression of this cytokine is essential to maintain a balance

between resolution of inflammation/infections versus immunopathology. Hence, the current study explores the role of two factors, (i) GAGs, in particular cell surface-DS (Chapter 4) and (ii) the anti-inflammatory cytokine, TGF-β1 (Chapter 5) in regulating IL-12 stimulated IFN-γ production in NK cells. Subsequent subsections introduce NK cells, IL-12 signalling and IFN-γ production.

1.6.1 Natural Killer (NK) Cells

NK cells are effector lymphocytes of non-specific, innate immunity that kill target cells including tumours and pathogen-infected cells (bacteria, viruses or protozoans), without prior immunization or exposure to foreign antigens (Vivier *Et. al.*, 2008; Morvan & Lanier *Et. al.*, 2016). They are large, granular lymphocytes, comprising 10-15% of peripheral blood lymphocytes and are also present in the spleen and bone marrow (Trinchieri, 1989). The importance of NK cells in the innate system is illustrated by rare human diseases in which NK cells are absent, defective or dysfunctional. Inspite of other active innate and adaptive immune components, NK cell deficiency is clinically manifested by frequent and severe infections and susceptibility to tumour development (Orange *Et. al.*, 2013). NK cells are attractive clinical targets due to their cytolytic and cytokine-producing capability. In recent years, NK cells have been gaining attention for their potential in cancer immunotherapies (Morvan & Lanier, 2016). Adoptive transfer of genetically engineered NK cells to *ex vivo* cultivation of NK cells in priming against tumour cell killing are attractive strategies in cancer treatment (Rezvani & Rouce, 2015; Granzin *Et. al.*, 2017). Based on these advances, a greater mechanistic understanding of NK cell responses is required to devise rational therapeutic strategies in the fight against infections, malignant transformation and autoimmunity.

NK cells function in innate immunity through two ways, target-cell killing and production of cytokines. To enable a prompt and an efficient innate response, NK cells express multiple activation molecules and receptors on their surface to recognise potential target cells (Smith *Et. al.*, 2000). Unlike T and B cells, NK cell receptors are not specific surface membrane TCR or immunoglobulin receptors. Indeed, they recognize the presence of altered MHC class I molecules on host cells which is a common consequence of viral infection or tumour transformation. Such an effector function in an MHC-unrestricted manner by NK cells distinguishes them from other

lymphocytes, importantly, T cells (Trinchieri, 1989; Vivier *Et. al.*, 2008). Thus, NK cells play a crucial role in immune surveillance and thereby innate anti-tumour immune responses.

NK cell activity is primarily triggered by cytokines such as interleukin-2 (IL-2), interleukin-12 (IL-12) (Trinchieri, 1998a) and IL-18 (Lauwerys *Et. al.*, 1999; W. Walker *Et. al.*, 1999) which are secreted by phagocytes or APCs in the early phases of infection (Borg *Et. al.*, 2004; Marcais *Et. al.*, 2013). Once activated, NK cells display spontaneous cytotoxic activity and rapidly secrete large amounts of chemokines, inflammatory cytokines-the most important of which is IFN-γ (Parihar *Et. al.*, 2002). This NK cell-produced IFN-γ is vital to the early control of microbial invasion before the involvement of T cells that are significant to adaptive cell-mediated immunity. *In vivo* studies support this, as SCID mice that lack mature T and B cells exhibit resistance to infections (Gazzinelli *Et. al.*, 1993; Tripp *Et. al.*, 1994), whereas, mice deficient in IFN-γ, or its receptor, showed increased susceptibility to a variety of pathogens, infections and tumour development (Schroder *Et. al.*, 2004). NK cell-produced IFN-γ has multiple biological effects (detailed in next section 1.6.2) that further aides in immune surveillance. Moreover, NK cell-derived IFN-γ amplifies the innate cycle of NK cell activation and shapes the development of subsequent and specific adaptive immune responses for the long-term control of pathogens or transformed cells (Moretta *Et. al.*, 2008; Cooper, Elliott *Et. al.*, 2009). For instance, NK cell activation on encounter with MHC class I ^{low} tumour cells was shown to stimulate DCs to produce IL-12. This was shown to further augment induction of $CD8^+$ T cell responses, however this T cell response was influenced by NK cell-produced IFN-γ which promoted Ag-processing and presentation by DCs activating $CD8^+$ T cells (Mocikat *Et. al.*, 2003). Thus, manipulation of NK cell activity by using cytokines is an important strategy in clinical settings against adaptive immune disorders such as graft transplants, autoimmune diseases and AIDS (Acquired Immuno Deficiency Syndrome) (Zwirner *Et. al.*, 2010, Morvan & Lanier, 2016).

Research work presented here employs a murine NK cell line, KY-1 (Karlhofer *Et. al.*, 1995), for experimental observations. KY-1 cells are more advantageous compared to primary NK cultures, as they provide an unlimited source of cells for mechanistic study and are endowed with stable or normal expression of cytokines upon activation. Studies conducted using these cell lines are highly reproducible and do not show the variability

associated with studies on freshly isolated primary cultures (Karlhofer *Et. al.*, 1995). All these characteristics render the KY-1 cell lines as very useful for mechanistic studies on cytokine signalling and regulation in NK cells (Garnier *Et. al.*, 2003).

1.6.2 IL-12 Signalled IFN-γ Production

IL-12 was first referred to as a natural killer cell stimulatory factor (NKSF) due to its ability to induce proliferation, cytotoxicity and IFN-γ production in NK cells from a culture of PBMCs (peripheral blood mononuclear cells) (Kobayashi *Et. al.*, 1989). Structurally, IL-12 is a heterodimer of 70kDa with subunits of 35kDa and 40kDa linked by two disulphide linkages (Podlaski *Et. al.*, 1992; Yuzhalin & Kutikhin, 2012). It is produced as a pro-inflammatory cytokine by APCs and phagocytic cells which includes macrophages, DCs and B cells, within hours of host invasion by microbial pathogens, viral infections (Chehimi & Trinchieri, 1994). The major physiological role of IL-12 is activation of NK cells and T cells to produce IFN-γ which is a central mediator of most IL-12-induced responses, as shown in Figure 1.9 (Hamza *Et. al.*, 2010; Vignali & Kuchroo, 2012). NK cells are most likely the first target cells by IL-12 to produce IFN-γ. An interesting microscopic study has shown temporal and spatial correlation between IL-12 production by DCs and IFN-γ production by NK cells in response to *Listeria monocytogenes* (LM) infection (Kang *Et. al.*, 2008) indicating IL-12 leads to crosstalk between DCs and NK cells stimulating innate responses. Moreover, in eliciting the production of IFN-γ, IL-12 is also known to synergise with a variety of cytokines like IL-2 (Wang *Et. al.*, 2000), IL-15 (Ni *Et. al.*, 2012) and IL-18 (Haeberlein *Et. al.*, 2010). In turn, IFN-γ further potentiates the activity of phagocytic cells in terms of microbial killing and stimulates more production of IL-12. Importantly, IL-12 and IFN-γ forms a positive feedback loop that represents the innate response to intracellular pathogens and inflammation (Grohmann *Et. al.*, 2001; Kang *Et. al.*, 2008).

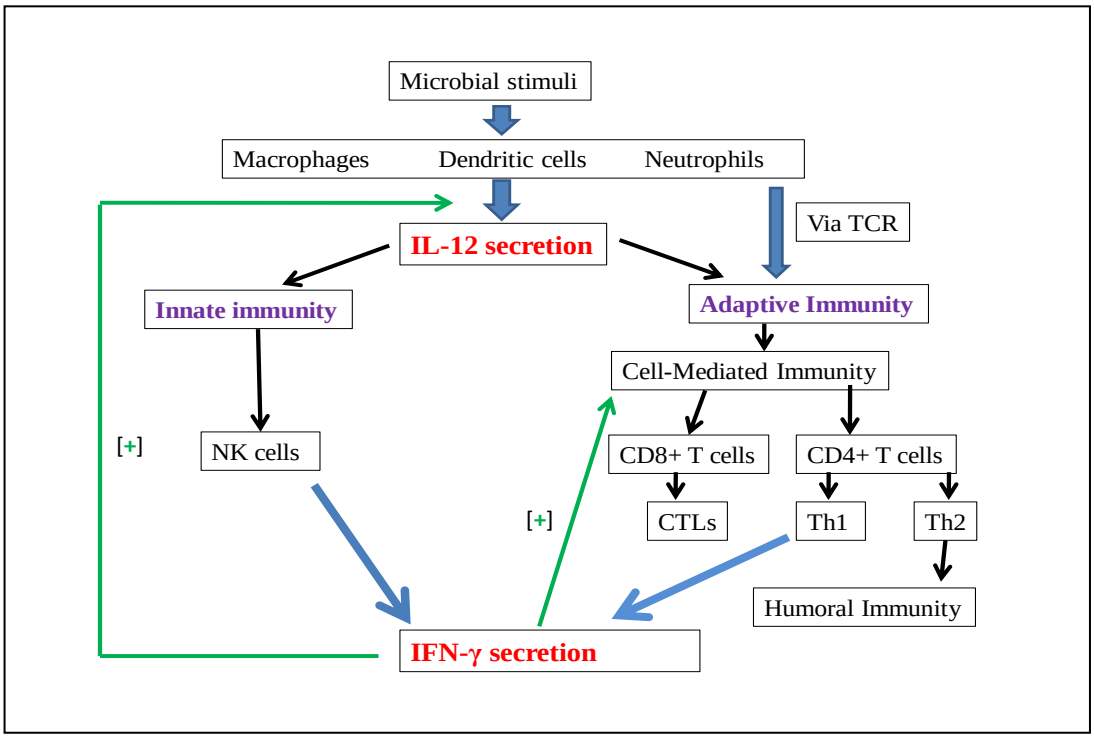


Figure 1.9: Biological role of IL-12 showing link between innate and adaptive immunity via IFN-γ: IL-12 produced by phagocytic innate cells such as macrophages, DCs and neutrophils in response to pathogens (bacteria, fungi, parasites and viruses) stimulates cytotoxicity and IFN-γ production of NK cells. This initial source of IFN-γ, firstly serves to positively amplify IL-12 secretion and the local inflammatory response to evade pathogens. Secondly, IFN-γ influences development of Th1 adaptive immunity initiated by IL-12 and indirectly inhibits Th2 or humoral immunity. Via TCR, IL-12 can directly and indirectly augment antigen specific adaptive immunity through generation of CTLs from naive $CD8^+$ T cells, and differentiation of naive $CD4^+$ T cells into IFN-γ secreting Th1 cells, together representing cell-mediated immunity. IL-12 offers resistance to infection, eradication of intracellular pathogens, and anti-tumour activity using both innate and adaptive mechanisms. Figure modified from (Romani, 1997; Szabo *Et. al.*, 2003; Lasek *Et. al.*, 2014)

Importantly, IL-12 is a key cytokine in polarizing T cells into IFN-γ secreting, T helper 1 (Th1) subsets (Mosmann & Coffman, 1989; J. Zhu & Paul, 2010), as illustrated in Figure 1.10. In contrast to NK cells, resting $CD4^+$ T cells do not express IL-12Rs. These naive $CD4^+$ T cells are activated via engagement of TCR with antigen-MHC II complexes presented by APC and co-stimulatory signals. Activation leads to production of the cytokines, IL-2 and TNF-α that initiate the expression of the IL-12R on $CD4^+$ T cells. IL-12 secreted by DCs initiates differentiation of naive $CD4^+$ T cells and directs them to develop into Th1 cells, which in turn, secrete IFN-γ. Together with IFN-γ, IL-12 further drives the balance between Th1 and Th2 cells towards the Th1 phenotype, also called cell-mediated immunity. Moreover, IL-12 alone can directly prime already differentiated, Ag-specific Th1 cells, independent of a TCR mechanism.

IL-12 can also stimulate development of IFN-γ-producing Th1 cells from resting memory T cells. Hence, IL-12 plays an essential role in bridging early non-specific, innate immunity and subsequent Ag-specific adaptive immunity via IFN-γ secretion (Trinchieri *Et. al.* 2003), as shown above in Figure 1.9.

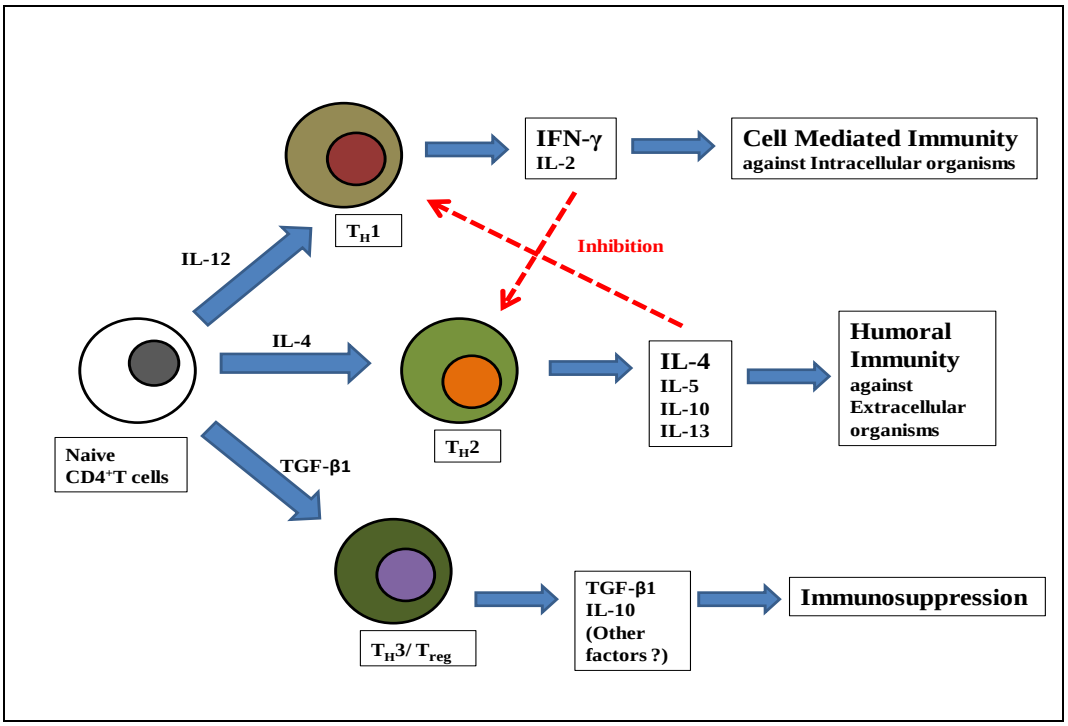


Figure 1.10: T helper cell differentiation: Cytokines are major inducers of T helper cell differentiation. IL-12 is an essential cytokine in the differentiation of naive $CD4^+$ T cells into IFN-γ secreting Th1 cells. IL-12 plays a central role in the initiation of cell-mediated immunity (Th1 development) involved in resistance to intracellular infections and further helps in the inhibition of humoral immunity (Th2 cells) against allergic reactions. TGF-β1 is an immunosuppressive cytokine secreted by another subset of Th cells called Tregs or Th3 cells and this regulates development of Th1/Th2 differentiation via Tregs. Figure modified from (Tato *Et. al.*, 2006).

On the other hand, differentiation of naive $CD4^+$ T cells in the presence of IL-4 results in Th2 effector cells which secrete a different profile of cytokines, consisting of IL-4, IL-5, IL-10, and IL-13 as shown in Figure 1.10. These Th2 cytokines are vital in allergic responses and help in the activation of B cells. This drives the immune response toward Th2-type immunity, also called humoral immunity (Ab-dependent immunity), which provides protection against extracellular pathogens, such as parasites (including helminths) and allergens. There is reciprocal inhibition of Th1 and Th2 pathways by cytokines secreted by each cell lineage. Specifically, IL-12 and IFN-γ blocks the secretion of IL-4 and IL-10 by Th2 cells. Whereas, Th2 cytokines block the secretion

of IFN-γ and hence, inhibit the development of cell-mediated immune responses by Th1 cells. Besides IL-10 (F. Ma *Et. al.*, 2015), TGF-β is another anti-inflammatory cytokine that negatively regulates IL-12 production and activity (M. O. Li *Et. al.*, 2006). In contrast to suppressing Th1 immunity, TGF-β promotes the development of regulatory T cells (Tregs), Th17 cells and other Th subsets that controls pro-inflammatory reactions following clearance of infection, thus maintaining immune homeostasis and tolerance (Sanjabi *Et. al.*, 2017).

Secretion of IFN-γ is indicative of the commitment to Th1 immunity. Although T cells are a major source of IFN-γ during infection, they require clonal expansion, differentiation into effector cells and they migrate to site of infection that takes several days to develop. Such Ag-specific IFN-γ production occurs over the course of time as part of an adaptive immune response. In contrast, activated NK cells secrete IFN-γ within hours of infection or cytokine stimulation (Cerwenka & Lanier, 2001; Stetson *Et. al.*, 2003). This innate source of IFN-γ *in vivo*, is a critical event during the early immune response whereas, IFN-γ produced from activated T cells in adaptive immunity is a secondary signal to boost the innate positive feedback loop of IL-12 production through activation of macrophages (Frucht *Et. al.*, 2001). Overall, this also forms the basis of the mechanism by which IL-12 acts as a potent vaccine adjuvant and activates several components of innate and adaptive immunity to play a role against infections (Wright *Et. al.*, 2008). Thus, the biology of IL-12 has been exploited in various therapeutic forms to stimulate cell-mediated immunity and a stable Th1 response in various disease settings including cancer (Tugues *Et. al.*, 2015) and Th2 disorders such as allergy or asthma (O'Garra & Murphy, 1996; Peterson *Et. al.*, 2003). Most IL-12-mediated anti-tumour effects have been shown to be dependent on IFN-γ production and its effector functions (Hess *Et. al.*, 2003). IL-12 gene therapy in mice was shown to inhibit liver metastasis of colon cancer cells. Moreover, this anti-metastatic effect of IL-12 mainly relied on NK cell activation and IFN-γ production (Uemura *Et. al.*, 2010). The therapeutic potential of IL-12 has been extensively investigated in the field of clinical oncology and is currently a focus of interest as an immunotherapeutic agent (Lasek *Et. al.*, 2014; Tugues *Et. al.*, 2015).

IFN-γ, once produced modulates immune responses. Besides supporting Th1 differentiation, functions of IFN-γ includes activation of macrophages to secrete tumour necrosis factor (TNF-α) and toxic forms of oxygen that destroy intracellular

microbes. IFN-γ is also directly involved in the expression of antiviral enzymes. It also promotes anti-tumour responses by increasing the expression of MHC class I molecules that aid in tumour surveillance and control (Dalton *Et. al.*, 1993; Agnello *Et. al.*, 2003; Schoenborn & Wilson, 2007). Collectively, IFN-γ favors the development of innate cell-mediated immune response as well as adaptive immunity. Based on these pleiotrophic effects of IFN-γ on various immune cells, this cytokine has been a useful therapeutic agent [as reviewed in (Miller *Et. al.*, 2009)].

Whilst the positive regulation of IL-12-induced IFN-γ production is advantageous, negative regulation is equally essential. In the case of overexpression of IFN-γ, destructive, or even lethal, inflammatory responses occur (Carson *Et. al.*, 2000). Excess IFN-γ has also been implicated in chronic inflammatory conditions and autoimmune diseases, such as insulin-dependent diabetes, multiple sclerosis, and systemic lupus erythematosus (SLE) (Schroder *Et. al.*, 2004; Lees, 2015). Also, IFN-γ increased lymphocyte recruitment generates secretion of more pro-inflammatory cytokines and such prolonged activation damages the inflammed tissues. Therefore, such a role of IFN-γ has been clinical target for therapeutic antibodies such as HuZaf and AMG811, as reviewed in (Miller *Et. al.*, 2009). Hence, understanding the molecular mechanisms that control IFN-γ production is of fundamental importance. In the following subsection, JAK-STAT pathway in IL-12 signalled IFN-γ production is described.

1.6.3 IL-12 Signalling via JAK-STAT Pathway

In the process of induction of IFN-γ, IL-12 signals through binding to its specific cell-surface receptor complex and this signal is transmitted via the intracellular signal transduction pathway called the JAK-STAT pathway (Janus kinase-signal transducer and activator of transcription) as illustrated in Figure 1.11 (W. J. Leonard & O'Shea, 1998; Hamza *Et. al.*, 2010).

The receptor for IL-12 has 2 subunits, IL-12Rβ1 and IL-12Rβ2 (Presky *Et. al.*, 1996). These receptors are pre-associated with the Janus family of protein kinases (JAKS) but itself do not have kinase activity. IL-12Rβ1 is required for high-affinity binding to the IL-12p40, a larger subunit of IL-12 and is associated with the Janus kinase family member, TYK2. IL-12Rβ2 recognises either the IL-12p35, a smaller subunit of IL-12 or heterodimeric IL-12. This receptor chain is associated with Janus kinase family

member, JAK2 that also mediates signal transduction. Both receptor chains are required to mediate maximal signalling (Sinigaglia *Et. al.*, 1999).

As seen in Figure 1.11, ligation of IL-12 receptors by IL-12 results in activation of JAK kinases that cross-phosphorylates the receptors at tyrosine residues of their cytoplasmic domain. Subsequently, the phosphorylated receptors are the recruiting sites for signal-transducing proteins. The IL-12Rβ2 chain contains three tyrosine residues that act as a docking site for a specific signal transducer and activator of transcription factor, STAT-4. The interaction occurs via the SH2 domain of STAT-4 with the IL-12Rβ2 chain resulting into tyrosine phosphorylation of STAT-4 at Tyr-693 by JAK2 (Yao *Et. al.*, 1999). This phosphorylation of STAT-4 is the key event in IL-12 signalling induced IFN-γ production (Thierfelder *Et. al.*, 1996; Visconti *Et. al.*, 2000; Wang *Et. al.*, 2015). On tyrosine phosphorylation, STAT-4 proteins are enabled to homodimerise and translocate into nucleus where they bind to specific sequences on the promoter and activate transcription of IL-12 inducible genes (Lammas *Et. al.*, 2000). Out of the 20 genes regulated, IFN-γ gene is the first gene to be induced by IL-12 and STAT-4-dependent signalling (Lund *Et. al.*, 2004). IFN-γ is also one of the most characterised target gene of STAT-4 that plays a key role in IL-12 induced Th1 differentiation (Good *Et. al.*, 2009).

STAT-4 knockout mice demonstrate the importance of JAK/STAT signalling in mediating the functional effects of IL-12. In mice lacking STAT-4, all the major functional effects of IL-12 on T and NK cells are markedly inhibited, including IFN-γ production and NK cell cytotoxic activity (Thierfelder *Et. al.*, 1996). Thus, activation of STAT-4 is an important control point in modulating IL-12 function in IFN-γ induction. Moreover, inappropriate activation of the JAK-STAT pathway contributes to oncogenesis and leukaemogenesis (Bowman *Et. al.*, 2000; T. S. Lin *Et. al.*, 2000); therefore, IL-12 activated STAT-4 is one of the attractive targets to leukaemia therapies. Study of agonism and antagonism of the IL-12 signalling pathway will provide novel strategies to enhance or weaken cell-mediated immune responses through effects on IFN-γ induction. Thus, it is important to study IL-12 signalling events that govern IFN-γ expression and production in NK cells.

1.6.3.1 STAT-4

As described above, STAT-4 is a critical mediator of IFN-γ production in the IL-12 signalling pathway (Good *Et. al.*, 2009; Wang *Et. al.*, 2015). The importance of STAT-4 is evident from various recent *in vitro* and *in vivo* studies that highlighted a key role of IL-12-signalled STAT-4 alone as well as in collaboration with other transcription factors in inducing IFN-γ production (Thieu *Et. al.*, 2008; J. Zhu *Et. al.*, 2012; Kurktschiev *Et. al.*, 2014). STAT-4 is activated by IL-12 alone or in synergy with other cytokines like IL-18 in the induction of IFN-γ (Lawless *Et. al.*, 2000). STAT-4 is also activated by IFN-α (S. S. Cho *Et. al.*, 1996; Athie-Morales *Et. al.*, 2004); however, this activation by IFN-α is only transient, whereas STAT-4 activation by IL-12 is long-lasting and enhances production of IFN-γ. On activation by IL-12, STAT-4 is phosphorylated at tyrosine 693 by JAK2 and TYK2 (Bacon *Et. al.*, 1995; S. S. Cho *Et. al.*, 1996). Indeed, mutation of Tyr 693 blocked IL-12 induced STAT-4 phosphorylation, transcriptional activity and IFN-γ secretion (Visconti *Et. al.*, 2000). Therefore, tyrosine phosphorylation is necessary for IL-12-induced STAT-4 homodimerization and DNA binding (Becker *Et. al.*, 1998; X. Chen *Et. al.*, 1998; W. J. Leonard & O'Shea, 1998).

Moreover, IL-12 also activates the p38/MKK6 pathway that leads to phosphorylation of STAT-4 on serine 721. Serine phosphorylation of STAT-4 is not required for its nuclear translocation and DNA binding activity; however, it is important to induce maximal transcriptional activity (Visconti *Et. al.*, 2000). Investigation of the biological importance of serine phosphorylation on STAT-4, reveal that it is critical for IL-12 induced IFN-γ production and Th1 development, but not for cell proliferation. Additionally, serine phosphorylation of STAT-4 was shown to be partially dependent on earlier tyrosine phosphorylation, whereas tyrosine phosphorylation of STAT-4 can occur even in the absence of serine phosphorylation (Morinobu *Et. al.*, 2002). Indeed, activated STAT-4 is known to bind directly to the *Ifng* locus and induce epigenetic changes leading to IFN-γ expression (Thieu *Et. al.*, 2008; Wei *Et. al.*, 2010). Therefore, it is important to study cytokine regulators of IL-12 signalling that inhibit STAT-4 phosphorylation and activation, resulting in reduced or abrogated IFN-γ production.

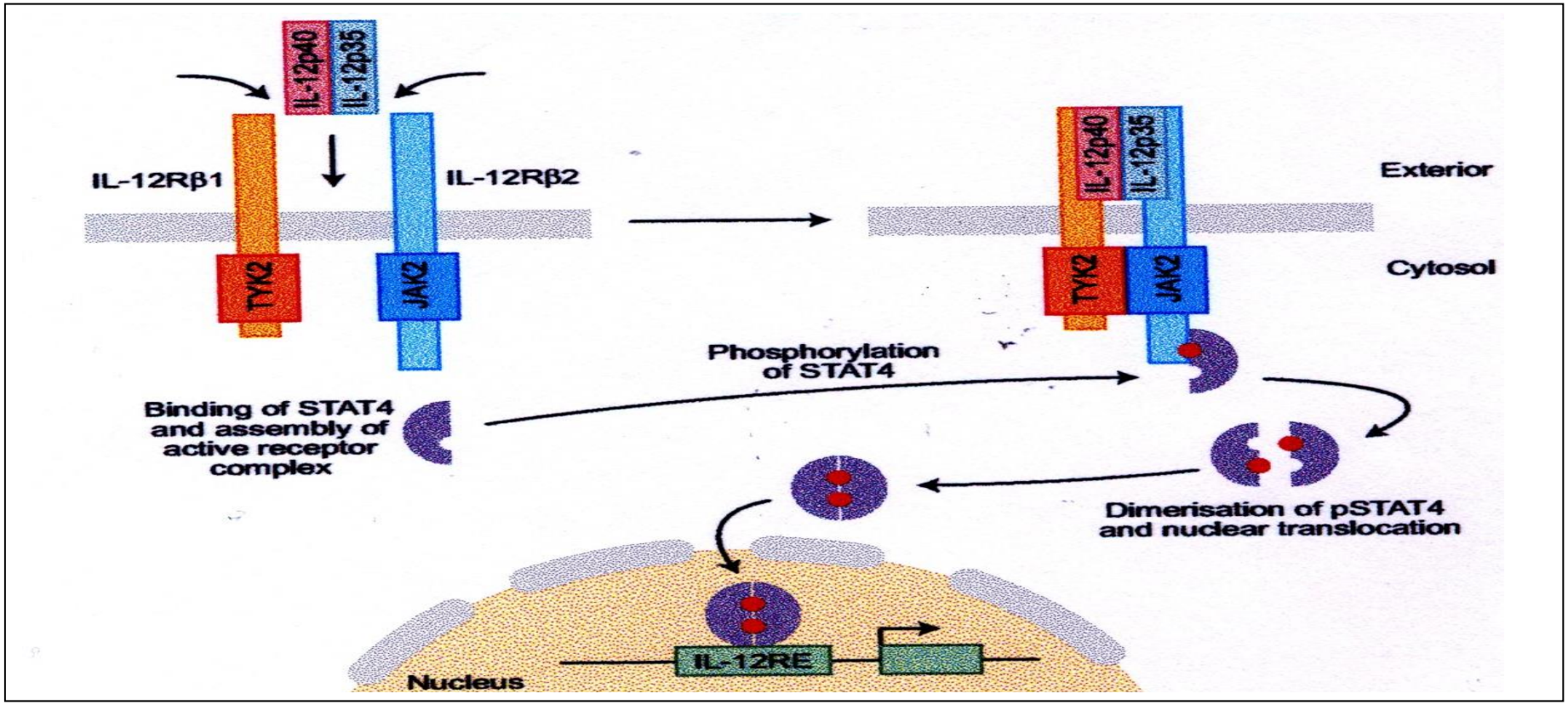


Figure 1.11 IL-12 signalling pathway: IL-12 activates the JAK/STAT pathway. Following binding of IL-12p40 and IL-12p35 to IL-12Rβ1and IL-12Rβ2, respectively, JAK2 and TYK2 are transphosphorylated. Subsequently, receptor chains are phosphorylated by activated JAKs. Phosphorylated IL-12Rβ2 serves as a docking site for STAT-4, which then itself is phosphorylated. STAT-4 homodimers translocate to the nucleus and bind to STAT-4 binding sites on the IFN-γ promoter to induce IFN-γ gene transcription. Reprinted from (Lammas *Et. al.*, 2000).

1.7 Role of Dermatan Sulphate (DS) in IL-12 Signalling Induced IFN-γ Production

As previously described (Section 1.2), within the immune system, GAGs control number of immune responses, mainly through activation of immune cells, cytokines and their activities (Davis & Parish, 2013; Coombe & Parish, 2015). However, the mechanisms by which GAGs affect these immune processes are less explored. There has been increasing recognition in the field to elucidate the molecular pathways regulated by GAGs. For instance, the role of decorin, a DS-PG, was shown in the modulation of delayed-type hypersensitivity responses, leading to reduced oedema and the concurrent reduction in the recruitment of $CD8^+$ T cells to the inflamed tissue using decorin KO (*DCN*$^{-/-}$) mice (Seidler *Et. al.*, 2011). Mechanistically, the absence of DS-PG from decorin KO fibroblasts and lymphocytes resulted in reduced expression of cytokines such as IFN-γ, TNF-α, CXCL-10 and their downstream effector molecules (Bocian *Et. al.*, 2013), indicating a role for DS chains in inflammatory responses. Studies by Dekker *Et. al.* also supports the importance of cell-associated DS in influencing IL-4 signalling in monocytes and thereby modulating expression of IL-4-mediated events (den Dekker *Et. al.*, 2008). Our laboratory previously observed that IL-12, like several other interleukins binds to heparin/HS and DS (Hasan *Et. al.*, 1999), but how these sulphated GAGs modulate IL-12 function, such as activation of NK cells, needs to be investigated. This section of the thesis seeks to investigate the underlying mechanism modulating IL-12 signalled IFN-γ production by NK cell-surface GAGs, particularly, DS (Chapter 4).

1.7.1 Dermatan Sulphate (Chondroitin Sulphate B)

Structurally, dermatan sulphate (DS/CS-B) is a sulphated GAG, consisting of a repeated disaccharide unit of [-4) GlcUA(*β*1-3) GalNAc(*β*1-]n, where GlcUA is glucuronic acid and GalNAc is *N*-acetylgalactosamine. DS belongs to the family of chondroitin sulphate (CS) due to the presence of GalNAc; however, GlcUA at most positions, along the chain is epimerised at C5 to IdoA (iduronic acid) (Trowbridge 2002; Sugahara *Et. al.*, 2003; Yamada & Sugahara, 2008). The presence of IdoA distinguishes DS from other members of chondroitin sulphate family (CS-A and CS-C). Nonetheless, IdoA residue is a common structural moiety in DS and heparin/HS. The functional significance of IdoA moiety has been demonstrated by the GAG chains

rich in IdoA to significantly inhibit proliferation of fibroblasts, than those chains containing higher amounts of GlcUA (Westergren-Thorsson *Et. al.* 1991). Therefore, the presence of this additional element makes DS a more important modulator of biological response than other CSs (Bao *Et. al.* 2004; Thelin *Et. al.*, 2013). DS-GAG chains linked to *O*-serine residues of the core protein forms DS-PGs. Role of DS either in the form of soluble or cell-surface PG has been appreciated in various biological responses such as cell growth and proliferation, coagulation, wound repair, infection, oncogenesis, and immune defense (as reviewed in detail by Trowbridge 2002; Malavaki *Et. al.*, 2008; Gubbiotti *Et. al.*, 2016).

1.7.2 DS in IL-12 signalling

Role of DS in immunoregulation has been observed through its binding affinity to various cytokines and growth factors (Mizumoto *Et. al.*, 2013; Gubbiotti *Et. al.*, 2016), however, there were no known reports regarding the involvement of DS in IL-12 signalling induced IFN-γ secretion until the work published by Garnier et al from our laboratory (Garnier *Et. al.*, 2003). Their work demonstrated a significant impact of DS-PG on IL-12-stimulated IFN-γ secretion by a combination of approaches that altered GAG expression on NK cells (Garnier *Et. al.*, 2003). Using range of GAGases, such as chondroitinase ABC, chondroitinase AC, hyaluronidase, and heparinitase I and II, they showed that the treatment with only chondroitinase ABC weakened the NK cell response to IL-12 to produce IFN-γ. This suggested a role of NK cell-surface DS-PG in the biological activity of IL-12. In a second approach, NK cells were cultured in the presence of 4-methylumbelliferyl-β-xyloside that inhibited IL-12-mediated IFN-γ secretion. 4-methylumbelliferyl-β-xyloside is a drug used to block GAG assembly onto PG chains and therefore abrogates PG synthesis (detailed in next Section 1.7.3). Moreover, the specificity of DS-PG involvement was confirmed when exogenously added, soluble DS had no effect on IL-12 activity in the presence of β-xyloside (Garnier *Et. al.*, 2003). Collectively, these observations with the use of chondroitinase ABC and β-xyloside indicated functional involvement of cell-associated DS chains in NK cell acitivty through a significant reduction in IFN- γ secretion in response to IL-12.

The involvement of DS, as implied in IFN-γ production (Garnier *Et. al.*, 2003), is distinct from the effects of other sulphated GAGs, including different forms of CS. Addition of soluble GAGs, like CS-A, CS-C, heparin, and HS at concentrations of 50

µg/ml to β-xyloside treated NK cells had no effect in either potentiating or restoring, β-xyloside suppressed IFN-γ secretion (Garnier *Et. al.*, 2003). Studies with primary cultures of NK cells have shown that these cells in the presence of β-xyloside, mainly produced CS/DS GAGs. This was ascertained by complete digestion of cell-associated and secreted culture material with chondroitinase ABC, suggesting NK cells do not synthesise heparin/HS (Christmas *Et. al.*, 1988). Additionally, studies conducted by Garnier *Et. al.*(2003) showed a lack of effect of heparinases on NK cell response to IL-12 which suggested either the absence of heparin/HS or that these GAGs are present but do not have a role in IL-12-induced IFN-γ production. Among CS, CS-A is specifically produced by NK cells but was observed to remain confined to secretory granules of the cells and only released on target cell lysis (Schmidt *Et. al.*, 1985). This was shown by the treatment of whole NK cells with chondroitinase ABC and such enzymatic treatment was unable to release the intracellular CS-A-PGs into the medium (MacDermott *Et. al.*, 1985). This eliminates the possibility of CS-A involvement in IFN-γ secretion. Therefore, these experimental findings along with observations from the Garnier *Et. al.* study suggested that DS is an essential requirement of IL-12 signalling in the induction of IFN-γ in NK cells. However, the mechanism of this DS-PG involvement in IL-12 signalling remains unelucidated. Importantly, this leads to the second goal of this thesis that investigates the underlying mechanism in IL-12-signalled IFN-γ production regulated by DS-PG.

In order to understand the mechanistic role of DS-PG in IL-12 signalling, various questions could be posed. Referring to Figure 1.11 of the IL-12 signalling pathway, the first question that arises is whether cell-surface DS-PG affects the initial step of IL-12-receptor binding? This would mean that DS-PG functions as a co-receptor that binds and presents IL-12 to its signalling receptor. Such an interaction has been described for monocyte cell-surface DS-PG which facilitates IL-4-induced signalling events via its receptor (den Dekker *Et. al.*, 2008). In the case of FGF-2, HS mediates cytokine-receptor binding and signal transduction (A. C. Rapraeger *Et. al.*, 1991; Yayon *Et. al.*, 1991). Supporting this co-receptor hypothesis is the finding that IL-12 is capable of binding to DS, albeit weakly, but not to CS-A or CS-C (Hasan *Et. al.*, 1999).

Further, the involvement of DS in IL-12-IL-12R interactions should reflect in changes in receptor-based phosphorylation of the IL-12-activated, specific transcription factor, STAT-4. Such an effect of DS-PG has been observed on the IFN-γ-induced signalling

pathway in endothelial cells, where the absence of DS-PG resulted in a significant reduction in IFN-γ-activated STAT-1 phosphorylation level and hence decreased downstream chemokine expression (Bocian *Et. al.*, 2013). On the other hand, does DS-PG indirectly interact with the components of IL-12 signalling to inhibit the events, downstream to STAT-4 phosphorylation? In this context, DS has been shown to increase expression of ICAM-1, a surface protein and its mRNA levels, independent of STAT-mediated signalling pathway in endothelial cells. In fact, DS-induced ICAM expression was initiated through activation of another transcription factor, NF-kB and its signalling pathway (Penc *Et. al.*, 1999). Therefore, in the current study, it is conceivable that DS might be involved in either transcriptional or translational control of IFN-γ expression.

Since Garnier et al's report demonstrated only partial inhibition of IFN-γ secreted from both β-xyloside and chondroitinase ABC treated NK cells (Garnier *Et. al.*, 2003), an alternative proposition for the underlying mechanism may be that DS interferes with the secretory pathway of IFN-γ. This implies that the role of DS-PG might be at the post-translational level, in extracellular secretion of IFN-γ protein. Such dependence on PGs at the post-translational level was suggested in the secretion of urokinase plasminogen activator, a protease from β-xyloside treated macrophages (Pejler *Et. al.*, 2003). In this context, chondroitinase ABC susceptible PGs have been suggested to be important for the golgi-associated intracellular transport of proteins to secretory granules in leukocytes (Lemansky & Hasilik, 2001; Galvin *Et. al.*, 1999). IFN-γ is a secretory protein that, once translated, follows normal ER-Golgi transport system. It has been shown that β-xyloside treated NK cells produce CS/DS GAGs (Christmas *Et. al.*, 1988) and IFN-γ is a GAG binding protein (Lortat-Jacob & Grimaud, 1991a). Perhaps, this indicates that IFN-γ protein remained GAG-bound and accumulated intracellularly. Therefore, obstruction in the secretion of IFN-γ and the consequent intracellular sequestration of this protein might be the reason for the low levels of IFN-γ protein detected from chondroitinase ABC or β-xyloside treated NK cells on IL-12 stimulation. Thus, this raises an interesting question whether DS-PG acts as an accessory molecule or component in IL-12 signalling that facilitates the IFN-γ secretion rather than its gene expression.

Broadly, to test these various hypotheses addressing the mechanism by which DS-PG affects IFN-γ expression in NK cells, a systematic approach was used in the current

study that either rules-in or rules-out, each of the posed hypotheses. Thus, this study should highlight a particular level of cellular regulation by DS-PG that would later enable us to focus on a specific area or level of regulation. With the substantial role of IL-12 in immune responses, this study which investigates the functional role of DS in biological activities of IL-12 would be a significant finding in immune regulation. Moreover, because IL-12 promotes Th1 immunity, implications may extend to the regulation of adaptive immunity at the Th1/Th2 cell dichotomy in context with cell surface and ECM proteoglycans.

1.7.3 Methods to Study the Functional Role of GAGs/PGs

Several methods are in use to investigate the GAG/PG dependence in cytokine activities, this includes removing the existing PGs from cell-surfaces with enzymatic treatment or blocking PG synthesis by use of chemical inhibitors. In the current study, β-xyloside (a chemical inhibitor) and chondrotinase ABC (an enzyme) are used to understand the involvement of DS in IL-12 signalling (Chapter 4). Subsequent subsections briefly describes each of the approaches used and their mode of action.

1.7.3.1 Use of β-xylosides as Chemical Inhibitors

β-xylosides are the most widely accepted tools used to define the physiological actions of PGs in the last 40 years (Okayama *Et. al.*, 1973; Fritz *Et. al.*, 1994b; Kuberan *Et. al.*, 2008; Persson *Et. al.*, 2016). β-xylosides contain D-xylose (Xyl) in β-linkage to hydrophobic aglycones such as *p*-nitrophenol, benzyl, estradiol, *p*-hydroxyphenol, or 4-methylumbelliferone as shown in Figure 1.12. The hydrophobic aglycone moiety aids in transport of the sugar molecule through the cell membrane and permeates to golgi bodies, where GAG synthesis takes place (N. B. Schwartz, 1977; Izumi *Et. al.*, 1994). As described earlier in section 1.3.2, in normal PG synthesis, sugar D-xylose is added to a serine residue of a core protein by enzyme xylosyl transferase forming 'xylosylated protein core' (Figure 1.12). Then, the enzyme galactosyltansferase I links (β1-4) the first galactose residue to this xylosylated protein core, followed by addition of another galactose unit in β1-3 linkage by enzyme galactosyl transferase II and a glucuronic acid moiety by glucuronyl transferase I (Gallagher, 2015; Prydz & Dalen, 2000; Prydz, 2015). This leads to the formation of a tetrasaccharide linkage (O-Ser-Xyl-Gal-Gal-GlcUA) on a PG core protein that initiates GAG chain synthesis and hence, PG

synthesis. However, when β-xyloside is added to the cells, they act as alternate substrates to endogenous xylosylated core proteins and competes with it for GAG assembly, resulting in disrupted PG synthesis. Indeed, β-xylosides act as exogenous primers stimulating the synthesis of GAG chains without the need for endogenous xylosylated core proteins and results in increased synthesis of free GAG chains and decreased PG synthesis.(N. B. Schwartz *Et. al.*, 1974a; Steward *Et. al.*, 1990).

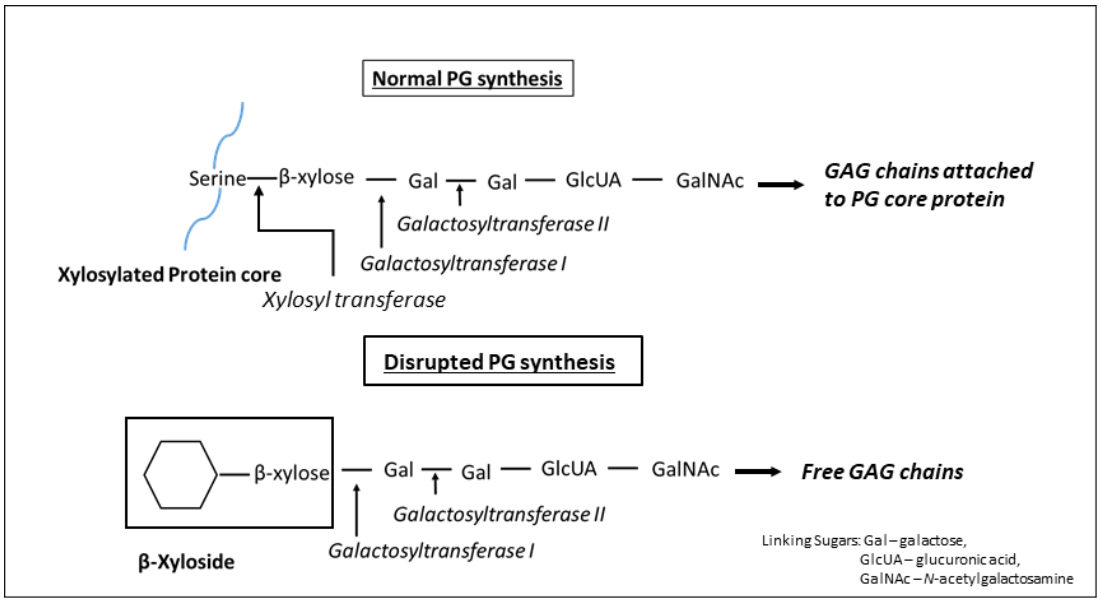


Figure 1.12 Mechanism of action of β-xylosides: A) Shows normal PG synthesis via the tetrasaccharide linkage that initiates GAG chain synthesis onto PG core protein **B)** Illustrates role of β-xyloside as an inhibitor of PG synthesis. β-xyloside contains β-xylose sugar attached to aglycone structure which substitutes for endogeneous xylosylated core protein. β-xyloside acts as a competitive acceptor ofsugar residues in the formation of a tetrasaccharide linkage that primes the synthesis of free GAG chains, thereby disrupting PG synthesis.

The most commonly employed β-D-xylosides include methylumbelliferyl-β-D-xyloside and *p*-nitrophenyl-β-D-xyloside. These β-xylosides cause disruption of PG synthesis and mostly prime synthesis of CS/DS GAG chains (N. B. Schwartz *Et. al.*, 1974b; Rosamond *Et. al.*, 1987; Coster *Et. al.*, 1991; Hahn & Birk, 1992; Fritz *Et. al.*, 1994b). However, the composition of GAGs produced on β-xylosides depends on the structure of aglycone (Lugemwa & Esko, 1991) and the concentration of β-xylosides used (Fritz *Et. al.*, 1994b). In most cases, β-xylosides have shown considerable selectivity in blocking CS/DS-PG synthesis (Hahn & Birk, 1992), with rather weak inhibition of HS-PG synthesis (Lugemwa & Esko, 1991; Fritz *Et. al.*, 1994b). This was seen regardless of the capacity of cells to produce these GAGs (H. C. Robinson & Lindahl, 1981; Sudhakaran *Et. al.*, 1981; Stevens & Austen, 1982). HS-GAG synthesis was shown to be selectively primed with high concentrations of *p*-nitrophenyl-β-D-

xyloside (A. Rapraeger, 1989) or when the aglycones of β-xylosides contain aromatic, polycyclic structures (2-naphthol and estradiol derivatives) (Lugemwa & Esko, 1991; Fritz *Et. al.*, 1994b; Belting *Et. al.*, 2002). This was thought to be based on the specificity of the enzyme in recognising the 'hydrophobic signal' which adds the first GlcNAc unit in heparin/HS synthesis (Lugemwa & Esko, 1991; Fritz *Et. al.*, 1994a; Fritz *Et. al.*, 1997). Thus, methylumbelliferyl-β-D-xyloside and *p*-nitrophenyl-β-D-xyloside are known primers stimulating the synthesis of CS/DS GAGs and inhibiting respective PG synthesis (Sobue *Et. al.*, 1987; Lugemwa & Esko, 1991).

The altered biosynthesis of GAGs/PGs using β-xylosides in several systems has suggested various roles of GAGs/PGs in cellular activities, metabolism, growth, differentiation, and development (Spooncer *Et. al.*, 1983; H. A. Thompson & Spooner, 1983; J. T. Gallagher & Hampson, 1984; Persson *Et. al.*, 2016). Rosamond *Et. al.* demonstrated with T lymphocytes that *p*-nitrophenyl-β-D-xyloside treatment completely inhibited synthesis of CSPG which resulted in decreased expression and activity of associated MHC class II molecules in antigen processing and presentation. However, T lymphocyte mediated target cell killing was unaffected by the presence of β-xyloside (Rosamond *Et. al.*, 1987). Similarly, T and NK cells, when cultured in the presence of β-xylosides exhibited 50% reduced PG production and released high amounts of CS-GAG chains into the medium (Christmas *Et. al.*, 1988; Steward *Et. al.*, 1990). However, in untreated T cells, free GAG chains were retained inside cells and formed ionic complexes with basic proteins within storage granules (Steward *Et. al.*, 1990). This indicated the role of endogenous GAGs in binding to basic proteins, such as in mast cells, where they form complexes with serine proteases (L. B. Schwartz *Et. al.*, 1982). Another example using β-xylosides showed that the secretion of proteinases like MMP-9 (matrix metalloproteinase-9) by macrophages was dependent on intact PGs, affecting synthesis at the post-translational level (Pejler *Et. al.*, 2003). Thus, β-xylosides have become common tools in the elucidation of the role of GAGs/PGs in the activation of cellular events.

1.7.3.2 Use of GAG-selective Enzymes

The enzymatic method has also been used in many studies to investigate the functional role of cell-membrane associated GAGs. Most commonly used GAG lyases include chondroitinases. Chondroitinases, or chondroitin lyases, are a family of enzymes that

depolymerise CS and DS galactosaminoglycans (Hernaiz & Linhardt, 2001). Chondroitinase ABC covers broad specificity for GAG substrates, including CS-A, CS-B, CS-C, and HA (Hamai *Et. al.*, 1997; Prabhakar *Et. al.*, 2005). The action of this enzyme results in disaccharide units and core protein. It is an exolytic enzyme that cleaves at the non-reducing ends of galactosaminoglycan chains (Figure 1.13). In spite of wide substrate specificity, chondroitinase ABC does not degrade keratan sulphate, heparin, and HS (Hamai *Et. al.*, 1997). Hence, use of this enzyme is most reliable in the preliminary studies or as supporting evidence to the involvement of specific GAGs/ PGs in any biological process. Recently, to identify the nature of GAGs chains synthesised in the presence of β-xyloside and their effect on breast carcinoma cells, various types of chondroitinases, including ABC were employed. The enzymatic treatment showed defined CS/DS chains exhibited cytotoxic effect on breast tumour cells (Persson *Et. al.*, 2016).

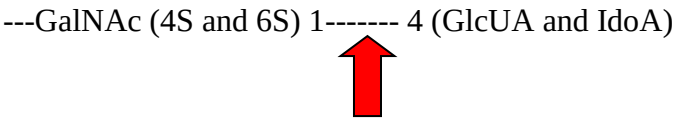


Figure 1.13 Mode of action of chondroitinase ABC

Chondroitinases have also been employed to show the importance of CS/DS GAGs in CNS biology (F. Li *Et. al.*, 2010). *In vivo* application of chondroitinase ABC at the site of CNS (Central Nervous System) injury promoted axon regeneration into damaged CNS tissue and reduced glial scar formation. This implied that the presence of CS/DS prevented reconstruction of damaged tissue by inhibiting axon transverse and neural outgrowth (Chau *Et. al.*, 2004; H. P. Li *Et. al.*, 2013). Similarly, *in vitro* studies have demonstrated various roles of CS/DS using chondroitinases. When human arterial smooth muscle cells were treated with chondroitinase ABC, the binding of IFN-γ to these cells was reduced by 50%. This significantly decreased the anti-proliferative effect of IFN-γ on cultured smooth muscle cells, suggesting that cell surface CS/DS-GAGs not only increased local IFN-γ concentration, but even modulated its biological activity (Hurt-Camejo *Et. al.*, 1999).

1.8 Role of TGF-β1 in IL-12 Signalling Induced IFN-γ Production.

Understanding the regulation of IL-12 effector functions during immune responses is the focus of most research interests because of the important implication of this cytokine in shaping adaptive immunity at the dichotomy of Th1/Th2 development. The balance between Th1/Th2 immune branches is the deciding factor in protective immunity against infections, as opposed to whether the response is appropriate and terminates without immuno-pathologies. Besides this fundamental role of IL-12 in enhancing Th1 cellular immunity, this pleiotropic cytokine with anti-tumour effects has gained central attention in the field of immuno-oncology (Lasek *Et. al.*, 2014; Tugues *Et. al.*, 2015). Importantly, intratumoural injection of an IL-12 gene (Ad-RTS-IL-12) is the most recent FDA approved therapy in clinical trials (2015) for the treatment of recurrent glioblastoma, a high-grade brain cancer (website link: http://ir.ziopharm.com/releasedetail.cfm?releaseid=923775). This therapeutic system is based on the controlled expression of IL-12 in cells by an orally taken activator ligand (veledimex pill). This therapy resolved the side-effects and toxicity associated with the systemic administration of recombinant IL-12 that for a long-time limited the beneficial, anti-cancer use of this cytokine.

Nevertheless, wider applications of IL-12 therapy in several animal tumour models and various clinical trials in cancer patients has shown a major setback due to immunosuppressive cytokines secreted by tumours as one of the immune evading mechanisms (Becker *Et. al.*, 2013). In recent years, an increasing number of studies have highlighted a critical role for Transforming Growth Factor-β1 (TGF-β1), as a suppressive cytokine produced by tumours to escape from host immunosurveillance (Flavell *Et. al.*, 2010; Tu *Et. al.*, 2014; Sanjabi *Et. al.*, 2017). Higher serum concentrations of TGF-β have been reported in cancer patients leading to immune dysregulation and associated with poor prognosis in several cancer types such as hepatocellular carcinoma, aggressive breast cancer and metastatic melanoma (Ikushima & Miyazono, 2010). Moreover, tumour growth was shown to be amplified by the co-infiltration of TGF-β1 secreting Tregs (T regulatory) cells along with suppressed adaptive immune cells such as CTLs and Th1 cells. The suppression of adaptive T cell responses was led by a downregulated innate signal from NK cell produced IFN-γ in the presence of TGF-β1 (Seo *Et. al.*, 2002; Flavell *Et. al.*, 2010). Naturally, TGF-β1 being an anti-inflammatory cytokine is the most effective inhibitor

of pro-inflammatory cytokines like IL-12, suppressing both its production from myeloid cells (macrophages, DCs) and effector functions on T and NK cells (Hunter *Et. al.*, 1995; Bellone *Et. al.*, 1995; Yu *Et. al.*, 2006; Lewis *Et. al.*, 2015). Hence, the negative regulation of IL-12 activity by counter-regulatory cytokines, as TGF-β1, helps to maintain immune tolerance and homeostasis by keeping a balance between the effective protection against pathogens and inflammatory shock due to IL-12 (M. O. Li *Et. al.*, 2006; Oh & Li, 2013; Sanjabi *Et. al.*, 2017).

Given the renewed interest in research for both these immunocytokines and in the efforts to unravel how TGF-β1 regulates IL-12 effector functions, the current study explores the mechanism for the inhibitory effect of TGF-β1 on IL-12-signalling induced IFN-γ production by NK cells (Chapter 5). Overall, this study will provide a better understanding of the regulation between these two key cytokines, TGF-β1 and IL-12, when present in an endogenous cytokine network, especially in a tumour microenvironment or in response to viral infections. Indeed, these cytokines are the therapeutic targets in regulating the efficient production of IFN-γ that is central in boosting anti-tumour or anti-viral immunity.

1.8.1 Transforming Growth Factor-β1 (TGF-β1)

TGF-β1 is a prototypical member of the TGF-β superfamily of cytokines that includes more than 35 related cytokines in vertebrates, such as TGF-βs, bone morphogenetic proteins (BMPs), Activins, Inhibins, Nodals, growth differentiation factor (GDF) and others that are crucial in regulating embryonic development and tissue homeostasis (Chen & Ten Dijke, 2016). TGF-β1 is one of the three highly conserved isoforms of TGF-β (TGF-β1, 2 & 3) described in mammalian cells, and they share considerable structural and sequence homology. These members constitute a TGF-β subfamily and have been recognised as powerful immunomodulatory agents exerting multiple effects on a wide spectrum of target cells. All three isoforms have similar properties *in vitro*, but TGF-β1 is the predominant isoform expressed in the immune system (Letterio & Roberts, 1998; Travis *Et. al.*, 2014).

The immunoregulatory role of TGF-β1 was first established in 1990 by the generation of TGF-β1 knockout mice. The phenotype of these mice revealed the suppressive activity of TGF-β1 and the requirement for a regulated immune response (Shull *Et. al.*,

1992; Kulkarni *Et. al.*, 1993). Mice deficient in TGF-β1 develop a widespread multiorgan autoimmune inflammatory disease and die within few weeks after birth (Kulkarni *Et. al.*, 1993). In 2000, development of mouse models with cell-type specific inactivation of TGF-β signalling revealed the regulatory network of the TGF-β pathway *in vivo* (Cazac & Roes, 2000; Gorelik & Flavell, 2000). Various transgenic mice were generated, whose T cells were specifically unable to respond to TGF-β1 and these developed either widespread autoimmunity (with somewhat slower kinetics than TGF-β1 knockout mouse) (Gorelik & Flavell, 2000) or pulmonary T cell pathologies (Nakao *Et. al.*, 2000), showing the importance of TGF-β1 in regulating immune tolerance. These observations demonstrated that TGF-β1 has a critical immunoregulatory and non-redundant function as an antagonist of Th1 development *in vivo*. The clinical relevance of this role of TGF-β1 was observed on the systemic administration of TGF-β1 that was effective in preventing Th1 cell-mediated autoimmune diseases of Experimental Allergic Encephalomyelitis (EAE) and Collagen-induced Arthritis (CIA) (Johns *Et. al.*, 1991; Kuruvilla *Et. al.*, 1991). To date, it has been almost 30 years of intensive research elucidating the immuno-regulatory and immuno-suppressive role of TGF-β1 in various pathophysiological conditions such as autoimmune diseases, inflammatory pathogenesis and infections (Gorelik and Flavell 2002b; M. O. Li *Et. al.*, 2006; Sanjabi *Et. al.*, 2017). However, the precise cellular and molecular mechanisms regulated by TGF-β1 in immune cell functions still remains less explored.

Innate immune cells such as NK cells are critical in viral infections and tumourigenesis. NK cells respond to various pro-inflammatory cytokines as IL-2, IL-12, or IL-18 alone, or in combination, to produce IFN-γ (Marcais *Et. al.*, 2013; Zwirner & Ziblat, 2017). Studies, specifically in NK cells demonstrated that TGF-β1 inhibited cell proliferation, cytokine production as well as cytolytic activity and hence suppression of all aspects of NK cell biology (Bellone *Et. al.*, 1995, Laouar *Et. al.*, 2005; Wilson *Et. al.*, 2011; Lewis *Et. al.*, 2015; Viel *Et. al.*, 2016). Studies also showed that TGF-β1 produced during viral infections limited NK cell functions, increasing susceptibility to virus persistence (Tinoco *Et. al.*, 2009). A well-designed study by Laouar *Et. al.* (2005) demonstrated an *in vivo* significance of TGF-β1 using a transgenic mouse model in NK cells. They specifically expressed the dominant-negative (dn) form of the TGF-β receptor II which resulted in blocking TGF-β1 signalling in NK cells. Consequently, increased amounts of IFN-γ production from NK cells was observed, leading to a Th1 type immune response and protection from *Leishmania major* infection. Using a similar

transgenic mouse model, more recent studies showed that a disrupted TGF-β1 response resulted in the generation of increased numbers of NK cells in these mice (CD11c-dnTGF-βRII), rather than DC-mediated functions. This indicated an essential role of TGF-β1 in the regulation of NK cell homeostasis and IFN-γ production in the early control of MCMV infections (Lewis *Et. al.*, 2015) and in Th1 cell development (Laouar *Et. al.*, 2005). Similar findings were reinstated in neonates showing CD11c-dnTGF-βRII infant mice were resistant to MCMV infections due to increased NK cell numbers and maturation (Marcoe *Et. al.*, 2012). Moreover, evidence from other studies support the role of tumour-derived TGF-β in weakening NK cell activity associated with IFN-γ release and cytotoxicity (Wilson *Et. al.*, 2011, Lindgren *Et. al.*, 2011). Nevertheless, an important question that still remains unaddressed from these studies is how the inhibitory effects of TGF-β1 are mediated on NK cell-produced IFN-γ. In view of the critical role of IL-12-induced NK cell IFN-γ in generating cellular immune responses against viral infections, intracellular pathogens and tumour development, understanding the cellular and molecular mechanism by which TGF-β1 regulates IL-12-mediated responses is important (M. O. Li *Et. al.*, 2006; Marcais *Et. al.*, 2013; Lasek *Et. al.*, 2014). Collectively, this prompted interest to investigate the underlying mechanism of signalling cross-talk between TGF-β1 and IL-12 in the molecular regulation of IFN-γ expression in NK cells which is the third and final goal of this thesis.

1.8.2 Crosstalk between TGF-β1 and IL-12 Signalling

Molecular understanding in the crosstalk between TGF-β1 and IL-12 signalling is less understood. Conceivably, one mechanism by which TGF-β1 interferes with the receptor-proximal signalling events induced by IL-12 in IFN-γ production could be via inhibition of the JAK-STAT pathway (Figure 1.14). Supporting this, Bright & Sriram (1998) have reported that TGF-β1 rapidly inhibited IL-12-induced phosphorylation of JAK2 and TYK2 by an unknown process in mouse T cells resulting in reduced STAT-4 phosphorylation. STAT-4 are cytoplasmic proteins which, on IL-12 stimulation, phosphorylate, homodimerise and translocate to the nucleus for target gene induction. In line with Bright et al's finding, Pardoux and colleagues demonstrated, using human T cells that downstream to STAT-4 phosphorylation, that TGF-β1 also inhibited the DNA-binding activity of STAT-4 (Pardoux *Et. al.*, 1999). In contrast to these findings, another study demonstrated that TGF-β1 neither inhibited IL-12 induced

phosphorylation of JAK2 and TYR2, nor phosphorylation of STAT-4 in activated human T and NK cells (Sudarshan *Et. al.,* 1999). Additionally, this study also showed that TGF-β1 did not inhibit nuclear translocation or DNA-binding activity of STAT-4. However, they still observed reduced IFN-γ transcripts correlating to suppressed IFN-γ production in the presence of TGF-β1. Taken together, it is evident from the above described studies that the effects of TGF-β1 on the IL-12-receptor based JAK-STAT pathway provided conflicting results. In the light of these contradictory findings, the role of TGF-β1 in the regulation of IL-12 signalling components in IFN-γ production is still not well understood. Collectively, there have been disagreements in the reports over the effects of TGF-β1 on receptor-proximal events of the IL-12-signalling pathway. Therefore, it is important to address this issue.

Further, downstream to receptor-based events, studies have reported impaired IFN-γ production by TGF-β1 that correlated to reduced IFN-γ mRNA levels, indicating transcriptional inhibition (Sudarshan *Et. al.,* 1999; Hayashi *Et. al.,* 2003; J. T. Lin *Et. al.,* 2005; Thomas & Massague, 2005). Interestingly, work led by Laouar and colleagues also demonstrated suppression of IL-12 or IL-12/IL-18 stimulated IFN-γ expression at both the mRNA and protein levels in TGF-β1 treated NK cells, whilst, NK cells from transgenic mice which expressed dn TGFRβ-II were resistant to such IFN-γ suppression. Thus, their findings suggest transcriptional regulation by TGF-β1 (Laouar *Et. al.*, 2005). Nevertheless, the molecular basis to this transcriptional control by TGF-β1 in NK cells is not well defined. Therefore, another level of crosstalk in IL-12/STAT-4 and TGF-β1/Smads signaling could be convergence at the transcriptional level, as illustrated in Figure 1.14. Smads are primary signal-transduction molecules in TGF-β signaling. One possible mechanism in transcriptional repression might be that TGF-β1 directly targets expression of key transcriptional factors important in IFN-γ expression. Besides STAT-4, one such key transcriptional factor is T-bet, which is markedly up-regulated by various cytokines, especially IL-12 and IL-18 in mature NK cells (Szabo *Et. al.*, 2002; Townsend *Et. al.*, 2004; Marcoe *Et. al.*, 2012; Leong *Et. al.*, 2017; Zwirner & Ziblat, 2017) and T cells (Thieu *Et. al.*, 2008; Oestreich & Weinmann, 2012; J. Zhu *Et. al.,* 2012). Significantly, TGF-β1 has been shown to downregulate T-bet expression in IL-12 stimulated $CD4^{+}$T cells (Gorelik *Et. al.,* 2002a). Moreover, retroviral transduction of T-bet into those activated T cells abrogated the inhibitory effects of TGF-β1 by inducing IFN-γ expression, as an indicator of Th1 differentiation (Gorelik *Et. al.*, 2002a). Consistently, NK cells from CD11c-dnTGF-βRII mice,

lacking active TGF-β signaling did show enhanced T-bet expression compared to WT derived NK cells during MCMV infection (Lewis *Et. al.*, 2015). Based on these observations, it is important to explore the role of T-bet in TGF-β1-regulated IFN-γ production by NK cells (Chapter 5). The next section, 1.8.2.1, briefly reviews what is currently known about T-bet expression in IFN-γ production and NK cells.

TGF-β1 initiates signalling via dimerization of transmembrane type I and type II receptor-associated serine/threonine kinases on the cell surfaces, as shown in Figure 1.14 (Massague, 2012). This allows Receptor II, a constitutive active kinase to phosphorylate Receptor I kinase domain that in turn acts as a Smad-binding site in phosphorylation of Receptor-specific Smads (R-Smads 2/3). Smads in TGF-β1 signalling are cytoplasmic proteins equivalent to STAT proteins in IL-12 signalling. Phosphorylated R-Smads interact with Common Smads (Co-Smad 4) to form Smad-complexes (2/3/4) which translocate to the nucleus to bind to a particular promoter region containing Smad-binding elements (SBEs), where they act as transcriptional regulators in the activation or repression of target genes. Moreover, these Smad-complexes have weak DNA-binding ability, so they associate or recruit other transcription factors that facilitates their binding to SBEs (Hata & Chen, 2016; Hill, 2016). Therefore, an alternative hypothesis in the signaling cross-talk between IL-12 and TGF-β1 could be that the TGF-β1-induced Smad complexes targets directly the cis-regulatory sites/elements on the IFN-γ promoter. Such binding elements for TGF-β1-signaled Smad-complexes (SBEs) are present on the proximal IFN-γ promoter region. The role of smad-complexes in transcriptional repression of cytotoxic genes such as *Gzm* B (granzyme B) has been demonstrated in $CD8^+$ T cells (Thomas & Massague, 2005). However, their direct role in IFN-γ promoter activity has not yet been established in NK cells. To explore this possibility, the current study seeks to determine the activity of the IFN-γ promoter in the presence of TGF-β1 in NK cells (Chapter 5). Indeed, the molecular targets and the mechanism by which TGF-β1 mediates IL-12-induced IFN-γ gene repression is not completely understood. Thus, in the current study IL-12 signalling pathway is dissected at various levels for investigating the interference by TGF-β1 in IFN-γ production.

1.8.2.1 T-bet

As mentioned above, besides STAT-4, T-bet is another key transcription factor in the regulation of IFN-γ production and Th1 development (recent review Leong *Et. al.*, 2017). T-bet stands for T-box expressed in T cells (product of gene Tbx21) and was first identified by Laurie Glimcher's laboratory in 2000, during the screening for new transcription factors specifically expressed in lineage-defined, Th1 cells (Szabo *Et. al.*, 2000). T-bet was detected as a positive regulator of IFN-γ expression not only in T cells but also in NKT, NK cells and B cells (Szabo *Et. al.*, 2000; Townsend *Et. al.*, 2004; Matsuda *Et. al.*, 2007). It was demonstrated that retroviral transduction of T-bet into either primary $CD4^+$ T cells, developing Th2 or effector Th2 cells, resulted in the induction of IFN-γ expression and simultaneous repression of Th2 cytokine expression (Szabo *Et. al.*, 2000). These findings suggested T-bet as a potent *trans*-activator of the IFN-γ gene and a master regulator of Th1 cell lineage commitment.

Moreover, T-bet-deficient mice exhibited a profound defect in the ability of $CD4^+$ T cells to produce IFN-γ with susceptibility to *L. major* infection and a marked *in vivo* shift of the Th1/Th2 balance towards the Th2 pathway (Finotto *Et. al.*, 2002; Szabo *Et. al.*, 2002; J. Zhu *Et. al.*, 2012). Although T-bet plays a significant role in $CD4^+$T cell differentiation (Szabo *Et. al.*, 2000), it is also involved in the effector functions of CD^+8 T cells against HBV and HCV infections (Sullivan *Et. al.*, 2003; Kurktschiev *Et. al.*, 2014), Ig class switching in B cells, and NK cell development, homeostasis and migration (Townsend *Et. al.*, 2004). Reduced numbers of NK cells in spleen, liver and peripheral blood were observed in T-bet-deficient mice (Townsend *Et. al.*, 2004). Moreover, NK cells that developed in the absence of T-bet resulted in immature NK cells (Yokoyama, 2004; Gordon *Et. al.*, 2012; Daussy *Et. al.*, 2014) with increased apoptosis and hence their reduced numbers in T-bet-deficient mice (Werneck *Et. al.*, 2008). Therefore, besides the developmental role of T-bet in NK cells, its absence leads to reduced life-span of NK cells *in vivo.* T-$bet^{-/-}$ NK cells also exhibit abnormalities in effector functions, such as low IFN-γ production and reduced cytotoxicity on stimulation by cytokines especially IL-12 and IL-18 (Townsend *Et. al.*, 2004). These observations suggested another role of T-bet in regulating the effector functions of NK cells in response to cytokines in the innate phase. Significance of this T-bet-dependent NK cell response was shown in initiating effective adaptive immune responses against

tumour antigens which was demonstrated by increased resistance of T-bet$^{-/-}$ mice from tumour metastasis on adoptive transfer of WT NK cells (Werneck *Et. al.*, 2008).

Signaling pathways involved in the induction of T-bet are still unclear. Studies with CD4^{+} T cells (Usui *Et. al.*, 2006) and CD8^{+} T cells (Y. Yang *Et. al.*, 2007) have shown that T-bet expression could be induced in a STAT-4 dependent and STAT-1 independent mode. However, contrasting these previous studies, recent evidence has established that in T cells, IL-12/STAT-4 and IFN-γ/STAT-1, signaling pathways can independently regulate T-bet expression (Oestreich & Weinmann, 2012; J. Zhu *Et. al.*, 2012). In NK cells, induction of T-bet expression was found to be independent of IFN-γ/STAT-1 signalling (Townsend *Et. al.*, 2004). IFN-γR deficient NK cells showed no impairment in T-bet expression, whereas STAT-4$^{-/-}$ NK cells showed a significant defect in T-bet induction on stimulation with IL-12 and IL-18, when compared to WT NK cells. These findings suggested a requirement for STAT-4 as an upstream signal that is important for T-bet induction in NK cells (Townsend *Et. al.*, 2004).

More importantly, T-bet has been identified as a regulatory target protein by TGF-β1 during Th1 development (Neurath *Et. al.*, 2002; Gorelik *Et. al.*, 2002a). TGF-β1 has been shown to negatively regulate IFN-γ expression and subsequent Th1 development, in a large part, by suppressing T-bet expression in T cells (J. T. Lin *Et. al.*, 2005; Park *Et. al.*, 2005) and NK cells (Yu *Et. al.*, 2006, Trotta *Et. al.*, 2008). Consistent with this, TGF-β1 suppressed T-bet expression was shown to control IFN-γ production in T cell-mediated inflammatory bowel diseases (IBDs) (Neurath *Et. al.*, 2002). Lin et al showed that TGF-β1 suppressed IFN-γ expression at Th1 priming with IL-12 through inhibition of STAT-4 expression. While at recall stimulation of Th1 effector cells, TGF-β1 uses a different mechanism that inhibits T-bet expression in IFN-γ suppression (J. T. Lin *Et. al.*, 2005). This suggests another mechanism under the control of TGF-β1 that is independent of STAT-4-regulated IFN-γ transcriptional activity and mediated by downregulation of T-bet. Because the expression of T-bet appears to be critical to IFN-γ production, this current study (Chapter 5) will examine T-bet expression in NK cells. Therefore, the direct involvement of T-bet in IFN-γ production in the presence of IL-12 and TGF-β1 will be explored. Although a number of different transcription factors have been suggested to participate in IFN-γ gene regulation (K. M. Murphy *Et. al.*, 2000), the current study focusses on two most important factors in IFN-γ expression, STAT-4 and T-bet. The susceptibility of STAT-4 and T-bet-deficient mice to infections

and resistance of these mice to autoimmune diseases further reinstates the critical significance of these two factors in IFN-γ production and Th1 functions (Thierfelder *Et. al.*, 1996; Mullen *Et. al.*, 2002; Szabo *Et. al.*, 2002; Thieu *Et. al.*, 2008; J. Zhu *Et. al.*, 2012; Kurktschiev *Et. al.*, 2014).

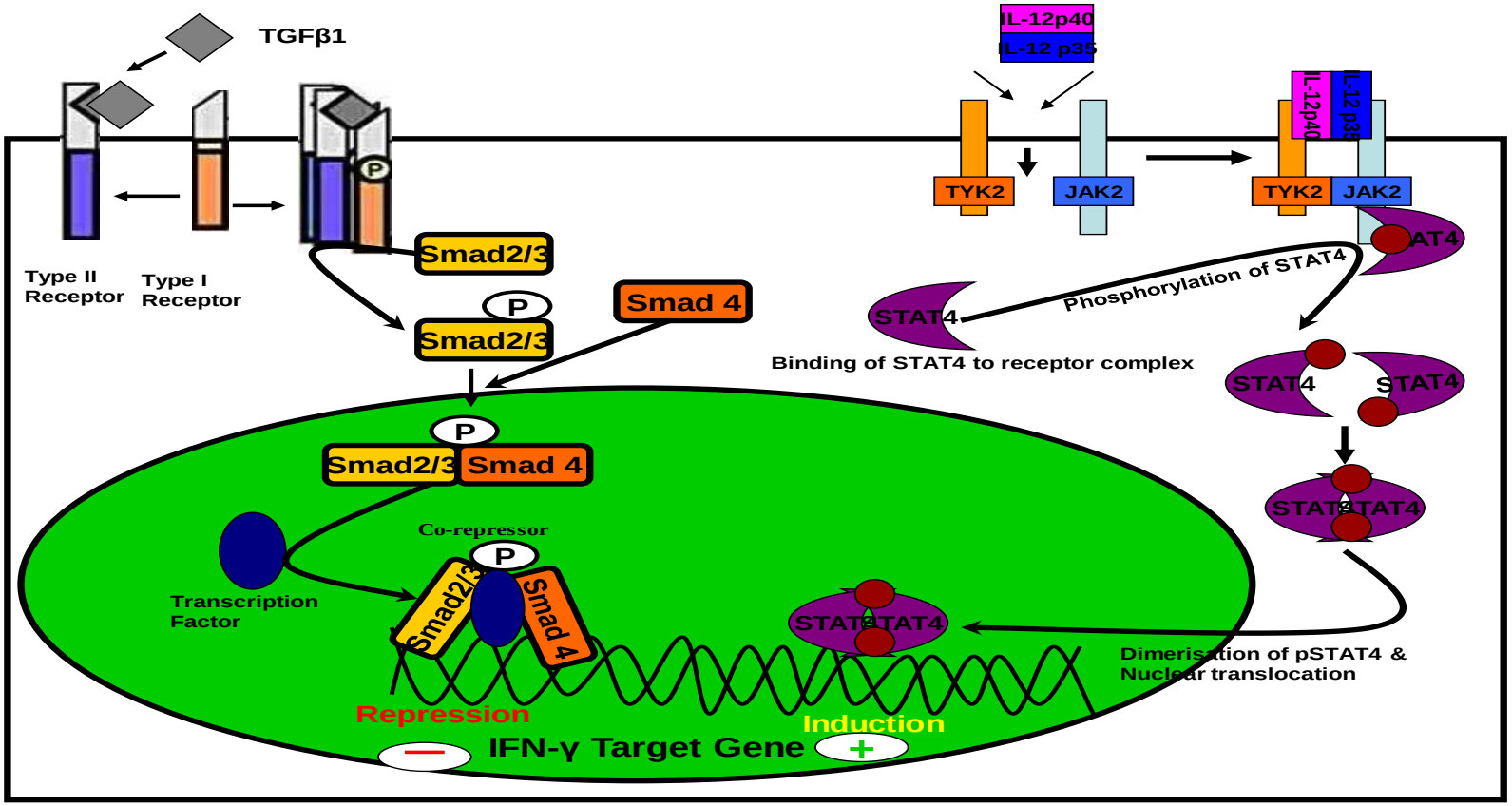


Figure 1.14 Hypothetical representation of a crosstalk between TGF-β1 and IL-12 signalling pathways regulating IFN-γ production in NK cells: IL-12 positively regulates IFN-γ production via activation of STAT-4 transcription factors. In contrast, TGF-β1 treatment activates transcription factors SMAD 2/3/4 in negative regulation of IFN-γ. Possible interaction between the components of these two signalling pathways in the repression of IFN-γ gene expression has been shown. (Figure modified and developed (from Massague & Chen, 2000a; Massague, 2012).

1.9 Research Aims and Objectives

In recent years, there have been significant advances in the study of cytokine-regulated NK cell activation. This knowledge could be used to devise rational therapeutic strategies in the fight against infection, malignant transformation, and autoimmunity. The overall aim of this thesis is to advance our understanding of the regulation of NK cell activities and thereby dichotomy of Th1/Th2 immune responses. As explained earlier (Section 1.1), in first part of the thesis, research work begins with a glycomics approach which integrates the role of GAGs in binding to cytokines. The second part of the research focuses on the modulation of a biological signalling pathway activated by specific cytokine, IL-12 in NK cells in the production of IFN-γ. Overall, there are three specific goals of the thesis. Their hypothesis and objectives are as described below.

1. Many cytokines belonging to structurally and biologically diverse families possess affinity for GAGs, particularly to heparin/HS *in-vitro*. Such affinity has often proved to be an indicator of a physiological importance. Therefore, an increasing number of cytokines have been investigated for their GAG-binding property. It could be hypothesised that a heparin-binding property is common to certain interleukins that share structural relationship with their respective IL family, members of which are well-characterised for their heparin-binding properties. The first specific goal of the thesis (Chapter 3) examines three interleukins, IL-11, IL-18 and IL-22 for their heparin-binding property. These three interleukins are yet to be examined for their heparin/HS binding interactions in this field of GAG-protein interactions in matrix biology. Interleukins, IL-11, IL-18 and IL-22 are enriched in basic amino acids, which are structural components of heparin-binding sequences. They also contain clusters of these basic residues in their primary sequences as required in the formation of heparin-binding domains. Our specific objectives are to determine (i) whether IL-11 binds to heparin as IL-6, both of which have similar biological activities and belong to gp-130 receptor cytokine family; (ii) whether IL-18 exhibits heparin-binding property like FGF family members that shares tertiary structural fold; and (iii) whether IL-22 is a heparin-binding cytokine as its structural and functional homologs, IL-10 and IFN-γ.

2. Previous work from our laboratory determined that stripping NK cells of a particular GAG, DS, with the action of chondroitinase ABC or a PG inhibitor, β-xyloside strongly inhibited IL-12 dependent IFN-γ secretion (Garnier *Et. al.*, 2003). From this, it could be hypothesised that NK cell-surface DS-PG may regulate IL-12 induced IFN-γ secretion. The second specific goal (Chapter 4) in this thesis is to investigate the underlying mechanism by which DS-PG is involved in IFN-γ secretion. To achieve this, the specific objectives are to determine (i) whether cell surface DS-PG directly affects IL-12/IL-12R binding or receptor activity as will be indicated by changes in phosphorylated levels of STAT-4; (ii) if receptor activation is not blocked, whether DS-PG indirectly regulates IL-12 signalling by interference in the later stages of STAT-4 phosphorylation leading up to the transcriptional, translational or post-translational level in IFN-γ expression; (iii) to deteremine the IFN-γ protein levels within the cells and secreted into the culture supernatant so as to indicate whether IFN-γ is sequestered intracellularly on removal of DS-PG. This objective would enable to distinguish a translational or post-translational from a transcriptional regulation by DS-PG.

3. Further, in the light of significance of NK cell-derived IFN-γ and IL-12 in the regulation of Th1 immune responses, the role of TGF-β1 was investigated. TGF-β1 often leads to down-regulation of cell-mediated/Th1 immunity (Gorelik & Flavell, 2000; J. T. Lin *Et. al.*, 2005; Sanjabi *Et. al.*, 2017). It suppresses the cytotoxic activity, cytokine production, and proliferation of NK cells (Bellone *Et. al.*, 1995; Laouar *Et. al.*, 2005; Lewis *Et. al.*, 2015). Although the immunosuppressive effect of TGF-β1 is being widely studied using NK and T lymphocytes, the molecular basis for these events are not clear. Further, conflicting reports in the literature on the signalling cross-talk between the pathways initiated by TGF-β1 and IL-12 in the regulation of IFN-γ production at the post-receptor level attracts the interest to address this issue. The third specific goal (Chapter 5) is to characterise the downregulation of IL-12 induced IFN-γ expression and secretion by TGF-β1 in murine NK cells. Therefore, the specific objectives in this study are: (i) to determine the anti-proliferative effect of TGF-β1 that might lead to suppression of IFN-γ; (ii) to determine the effect of TGF-β1 on the key event in IL-12 signal transduction pathway i.e. phosphorylation of STAT-4; and (iii) to detect the expression of an important

transcription factor in IFN-γ expression, T-bet in the presence of TGF-β1 (iv) to demonstrate the inhibitory effect of TGF-β1 on IFN-γ promoter activity. Thus, we unveil interplay of regulatory mechanisms between pro-inflammatory cytokine IL-12 and anti-inflammatory cytokine TGF-β1 in curtailing IFN-γ production.

CHAPTER 2

MATERIALS AND METHODS

2.1 Heparin Binding ELISAs

A Nunc Maxisorp 96-well plate (Life Technologies, Paisley, Scotland) was coated with 100 µl of Tris-HCl buffer (50mM, pH 7.4) containing either 25 ng of newly synthesised heparin-BSA conjugate (heparin-porcine intestinal mucosa, sodium salt, grade I-A from Sigma-Aldrich Co., Poole, UK) or the same amount of mock-conjugated BSA (BSA-99% purity, Sigma-Aldrich Co., Poole, UK) (Section 2.1.1) and incubated overnight at 5^{o}C. The wells were then washed three times with 400 µl phosphate buffered saline (PBS, pH 7.4) and blocked with 200 µl per well of Blocking buffer [PBS containing 1% BSA (w/v)] for 15 minutes at room temperature (R.T.) on a rotating platform. After washing the plate three times with PBS, 100 µl of respective cytokines dissolved in Tris-HCl buffer (50 mM, pH 7.4) were incubated for 2 hours.

Following incubation, the plate was washed three times with PBS-T [PBS with 0.05% tween 20 (v/v)] and incubated with specific primary antibody diluted in blocking buffer (1:200), for 2 hours (see Table 2.1 for list of cytokines and specific primary antibodies used for heparin-binding ELISAs). Subsequently, the plate was washed three times with PBS-T and incubated with the secondary antibody diluted in blocking buffer (1:1000) [Alkaline phosphatase conjugated polyclonal Rabbit anti-Goat Immunoglobulins (IgG) or polyclonal Goat anti-Rabbit IgG (Sigma-Aldrich Co., Poole, U.K.)] for 30 minutes. The plate was washed five times with PBS-T and 100 µl per well of p-nitrophenol phosphate solution (para-nitrophenol phosphate FAST tablets, Sigma-Aldrich Co., Poole, U.K.) was added as a substrate to the enzyme activity. After 20 minutes of incubation at room temperature on a rotating platform for colour development, 50 µl of stop solution (2N H_2SO_4) was added. Phosphatase activity was detected by measuring the absorbances at 405nm on an E_{max} microtitre plate reader (Molecular Devices Corp., Sunnyville, CA, USA).

Table 2.1: List of cytokines and primary antibodies used for heparin binding ELISAs

Cytokines	**Origin**	**Supplier Details**	**Primary Antibodies**	**Supplier Details**
GDNF	human	R&D Systems (Abingdon, U.K.)	Goat anti-human GDNF polyclonal Ab	R&D Systems (Abingdon, U.K.)
IL-11	human	R&D Systems (Abingdon, U.K.)	Goat anti-human IL-11 polyclonal Ab	R&D Systems (Abingdon, U.K.)
IL-18	human	MBL Systems (Japan)	Rabbit anti-human IL-18 polyclonal Ab	Santa Cruz Biotechnology Inc. (C.A., USA)
IL-22	human	R&D Systems (Abingdon, U.K.)	Goat anti-human IL-22 polyclonal Ab	R&D Systems (Abingdon, U.K.)

2.1.1 Synthesis of Heparin-BSA Conjugate

The immobilised form of heparin to be used in the ELISA was obtained by a chemical coupling reaction that employed sodium cyanoborohydride in the synthesis of the heparin-BSA conjugate, as previously described (Najjam *Et. al.*, 1997a). The reaction coupled heparin chains by their reducing ends to BSA. The reaction products, containing high M_r complexes were separated from uncomplexed BSA and heparin by gel filtration using Sepharose 4B.

The fractions eluted from the Sepharose 4B column were analysed for protein-BSA using coomassie blue, and heparin using 1, 9-dimethylmethylene blue reagent. The analysis of collected fractions for protein and heparin were as indicated by absorbances at 525nm and 595nm respectively (Figure 2.1A). The column fractions within the inset (Figure 2.1A) representing the upward trend of the curve were labelled 1 to 8 and further analyzed by polyacrylamide gel electrophoresis against standard heparin or BSA. The gel was stained for detection of heparin by Azure A staining (Figure 2.1B) and BSA by silver staining (Figure 2.1C).

Both the resolving gels shown in the Figure 2.1B and 2.1C contained excluded material which indicated the presence of high M_r components that were unable to penetrate the gel. This excluded material represented the heparin–BSA conjugate. A slow migrating smear of material between the excluded material and the stained band for free heparin or free BSA also represented heparin-BSA conjugate, but of smaller size. It penetrated the gel as seen in Figure 2.1B and Figure 2.1C and contained a mixed size of complexes of heparin chains coupled to BSA. Fractions 2-4 containing both the forms of heparin-BSA complex were selected and pooled together as a single heparin-BSA complex preparation for use in ELISA experiments. Although the pooled fraction was highly concentrated with heterogenous heparin-BSA conjugate, it also contained small amounts of uncomplexed heparin and BSA. However, later fractions, from 5 onwards were seen to contain some amount of this complex material in addition to appreciable quantities of free heparin and BSA and were therefore, excluded.

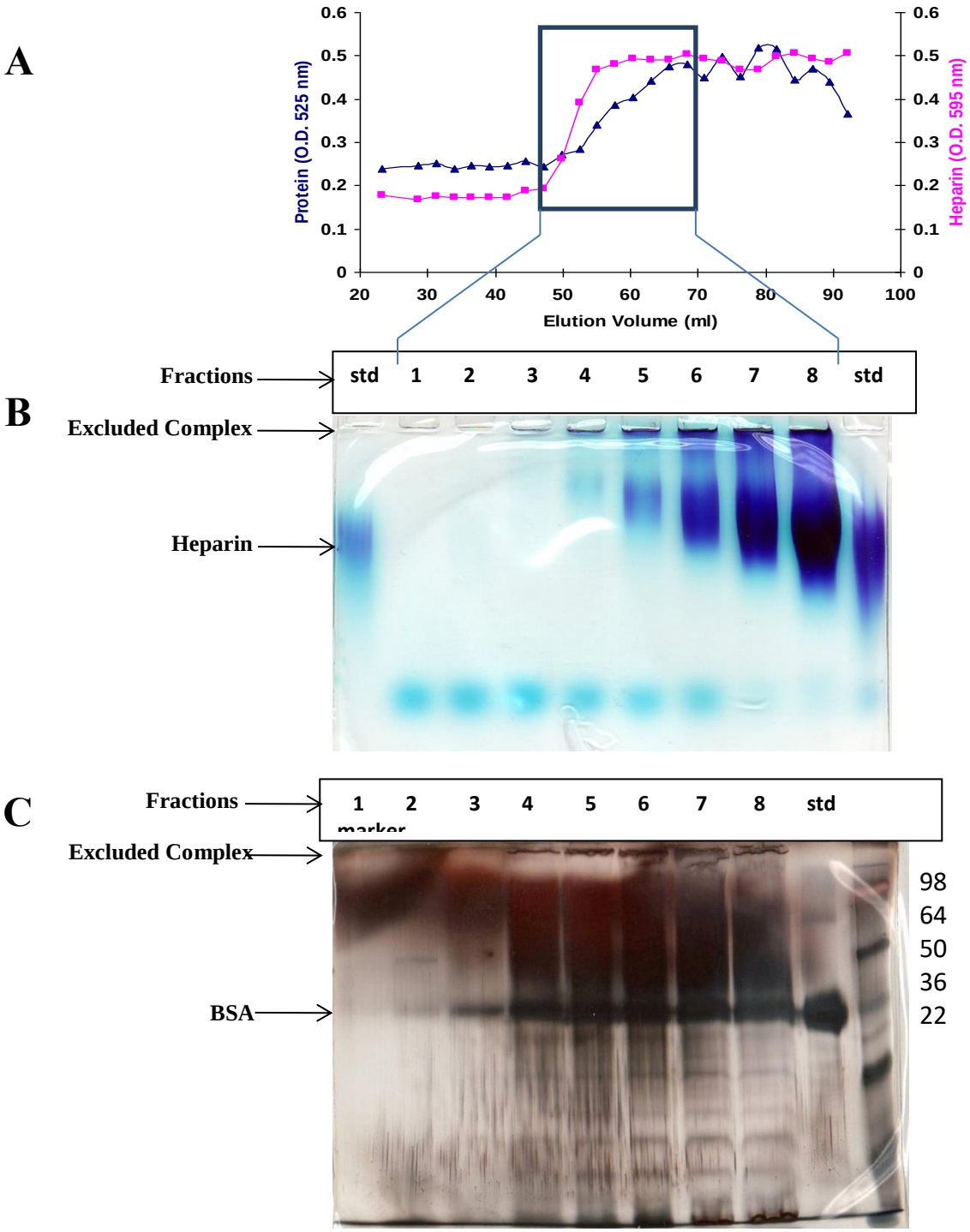


Figure 2.1: Synthesis of heparin-BSA conjugate: The dialysed reaction mixture was eluted through a 1 x 85 cm column of Sepharose 4B at a flow rate of 12 ml/hr. **A)** Elution profile of fractions collected (~ 2.5ml) and assayed for protein (▲) and heparin (■). Fractions 1-8 (inset), representing elution volume 50-65ml, were subjected to **B)** GAG electrophoresis of collected fractions with std as heparin (2.5µg and 5µg per well) was run on 15% polyacrylamide gels stained with Azure A, and **C)** Protein SDS-PAGE with silver staining, in order to visualise heparin and protein migration respectively with std as BSA (1.25µg per well).

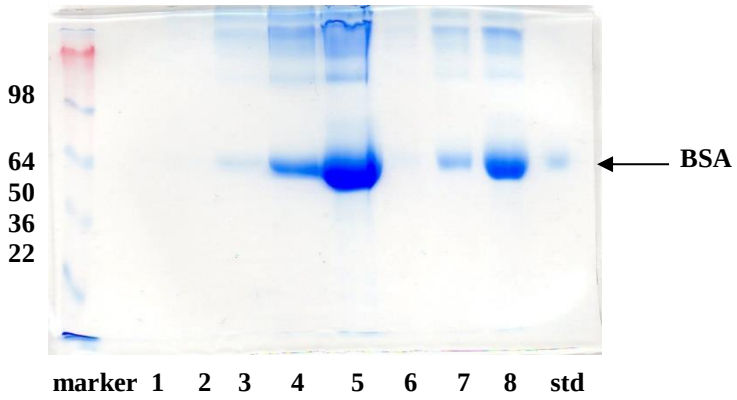


Figure 2.2: SDS PAGE for mock-conjugated BSA: Gel shows various fractions 1-8 resolved on SDS-PAGE of BSA treated under identical conditions as conjugate and stained by coomassie blue. Std is a pre-conjugate sample-BSA.

As a control, mock-conjugated BSA was similarly resolved by gel filtration. Fractions obtained were labelled 1-8, as shown in Figure 2.2 and were analysed by polyacrylamide gel electrophoresis. The fractions 2-4 were selected as pooled fraction representing mock-conjugated BSA. The protein concentration of the pooled fractions for heparin-BSA conjugate after lyophilisation and redissolving in buffer was quantified as 157 ng/µl (fractions 2-4 from Figure 2.1B and 2.1C). The concentration of mock-conjugated BSA was determined as 295 ng/µl (fractions 2-4 from Figure 2.2).

2.2 KY-1 Cell Line: Culture, Maintenance and Storage

KY-1 cells were NK cells obtained from $p53^{-/-}$ (tumour-suppressor gene) mice, which confer immortality to these clones as tumour cell lines. However, they require IL-2 as a factor for *in vitro* growth and proliferation as described in (Karlhofer *Et. al.*, 1995). This cell line, characterised by FACS (Fluorescence-Activated Cell Sorting) for the expression of NK1.1 and Fc receptor was received from the laboratory of Dr. Wayne Yokoyama (Washington University, School of Medicine, St. Louis, MO). Frozen vials of KY-1 cells were thawed at R.T for 5 minutes followed by thawing at 37 ^{o}C in water bath with occasional and gentle swirling. All the vial contents were transferred to 10ml of growth media (RPMI-1640, Sigma-Aldrich Co., Poole, U.K.) supplemented with 10% Foetal Calf Serum (FCS) (Invitrogen Ltd, Paisley, UK), 10mM glutamine

(Invitrogen Ltd, Paisley, UK), 100µg/ml penicillin/streptomycin (Invitrogen Ltd, Paisley, UK) and 50µM 2-mercaptoethanol (Sigma-Aldrich Co., Poole, U.K.). The cell-suspension was spun at 1000 rpm for 3 minutes, cells were re-suspended in fresh 5ml growth media and cell viability was determined by Nigrosin staining method (Section 2.6). Cells were counted using an improved Neubauer Haemocytometer and plated with a density of 2 x 10^5 cells/ml of growth media with a total volume of 5ml in a 25cm^2 flask (T25) with vented caps (Orange Scientific, B-1420 Braine-I' Alleud, Belgium). Recombinant human IL-2 (rhIL-2) (R&D Systems, Abingdon, U.K.) at a concentration of 37.5 ng/ml was added to the cells and the T25 flasks were incubated at 37^{o}C, 5% CO_2 and 95% relative humidity for 2-3 days. The KY-1 cells are mostly adherent cells with few cells growing in suspension. Their growth rate seems to improve with slightly higher density of plating (personal communications from Dr. Yokoyama's laboratory). Approximately 10% cell viability was observed with freshly thawed cells.

For continuous maintenance of these KY-1 cells in laboratory, they were cultured as a monolayer in a T25 flask with growth media supplemented with rhIL-2, in a 37^{o}C incubator with 5% CO_2 and 95% relative humidity as mentioned above. Once the cells reach 70-80% confluency, which is in approximately 3-4 days, the supernatant media was aspirated and the monolayer was treated with cell dissociation buffer (Invitrogen Ltd, Paisley, UK) to detach adherent cells. Once detached, 10ml of growth media was added to cell suspension and the contents were transferred to a 50ml falcon tube containing 30ml of growth media without IL-2 and spun at 1000 rpm for 3 minutes. The cell pellet obtained was re-suspended in 5 ml of growth media and cells were counted before re-seeding, using an improved Neubauer Hemocytometer. Cell viability was determined by Nigrosin staining method and cells were plated with a density of 1 x 10^5 cells/ml in a T25 flask containing a total volume of 4ml growth media supplemented with IL-2. When scaling-up was required, cells were cultured in 75cm^2 (T75) flasks and cell density was proportionately calculated.

For long-term storage of KY-1 cells, a cell suspension of 1 x 10^7 cells/ml was prepared in 100% sterile FCS. To that an equal volume of 20% DMSO in FCS (v/v) was added drop by drop so that the final DMSO concentration becomes 10% v/v. This cell suspension was mixed well and 0.5 ml dispensed into individual cryovials to be kept overnight at -80^{o}C in a Nalgene freezing unit (Thermo Fisher Scientific, Rockford,

USA). Next day, the cryovials were transfered to liquid nitrogen (-196 ^{o}C) for long-term storage.

2.3 Stimulation of KY-1 Cells with IL-12

For the experiments described in Section 4.2 & 5.2, KY-1 cells were used as an *in vitro* model for rmIL-12 stimulated IFN-γ production in NK cells. KY-1 cells were stimulated with rmIL-12 according to the modified protocol from Garnier et al, (Garnier *Et. al.*, 2003). Confluent T25 flasks with 70-80% of adherent cells were detached using 2.5 ml of cell dissociation buffer and washed in 40 ml of growth media. The cells were resuspended in the same media to give 5 x 10^5 cells/ml without adding IL-2. 0.5 ml/well of this cell suspension was used to set up a 24-well plate. For assays requiring 6, 12 and 96-well plates, cell numbers and volumes added per well were proportionately calculated to the surface area of the well. Increasing concentrations of IL-12 from 0 to 250 pg/ml at the interval of 50 pg were added to the wells. The plate was incubated overnight (16 hrs) at 37^{o}C with 5% CO_2. At the end of the incubation period approximately 250 µl of culture supernatant was collected, spun at 1000 rpm/5 minutes to obtain cell-free supernatant samples. Samples were used to detect IFN-γ secreted by KY-1 cells by ELISA (Section 2.5).

2.3.1 Addition of α/β-xylosides

The effect of various xylosides such as 4-methylumbelliferyl-7-β-D-xyloside, p-nitrophenyl-β-D-xyloside, p-nitrophenyl-α-D-xyloside and p-nitrophenyl-β-D-galactoside (all from Sigma-Aldrich Co., Poole, U.K.) was determined on IL-12-stimulated KY-1 cells (150 pg/ml of IL-12) (Section 4.2.6). Due to the lipophilic nature of these xylosides, they were dissolved in DMSO solvent (Sigma-Aldrich Co., Poole, UK). As an internal control in these experiments, DMSO alone, without xyloside was added to the cells at the same final concentration (v/v) as DMSO with xyloside. After treatment with respective xyloside and the required incubation time, cell-free culture supernatant was collected and assayed for secreted IFN-γ by ELISA (Section 2.5)

In some experiments (Section 4.2.5), the effect of xylosides was studied on cellular IFN-γ, that is IFN-γ protein accumulated within the cells. For this, the supernatant media from xyloside treated and IL-12-stimulated KY-1 cells was aspirated and the

adherent cells were washed with 350 µl/well of ice-cold TBS buffer pH 7.4. Cells were then lysed by addition of cold lysis buffer which is 1% v/v Triton X-100 in TBS buffer pH 7.4 containing 0.1% protease inhibitors cocktail (Sigma-Aldrich Co., Poole, U.K.). On addition of lysis buffer, cells were scraped, and lysate was pipetted several times to break up cellular clumps to release intracellular proteins (Bacon *Et. al.*, 1995; Sudarshan *Et. al.*, 1999). The contents of each well was collected in 0.5ml Eppendorf tubes and incubated on a roller at 4 ^{o}C for 20 minutes, followed by cold spin for 15 minutes at a maximum speed of a table-top centrifuge. The cell lysate was collected and cellular IFN-γ protein was detected by ELISA (Section 2.5).

2.3.2 Addition of Chondroitinase ABC

Chondroitinase ABC enzyme (E.C.4.2.2.4) used was a protease free enzyme obtained from *Proteus vulgaris* (Seikagaku Corporation, Tokyo, Japan). This lyophilised enzyme containing 20 mM Tris buffer pH 7.2 was reconstituted in 200 µl of enzyme buffer (prepared by mixing 0.2% BSA with 50% v/v glycerol) to get a final concentration of 10 U/ml. As per the product datasheet, 1 unit (U) is defined as the quantity of the enzyme that catalyses the formation of 1 µmole of unsaturated disaccharide from chondroitin sulphate-C per min at 37^{o}C and pH 8.

To determine the effect of enzyme chondroitinase ABC on IFN-γ production (Section 4.2.2), 0.1 U/ml of this enzyme was added with IL-12 (150 pg/ml) to KY-1 cells. As an internal control, equivalent amount of enzyme buffer was added with IL-12 and the plate was incubated overnight at 37^{o}C and 5% CO_2. Cell-free culture supernatant was collected and assayed for IFN-γ by ELISA (Section 2.5).

2.4 Assay for detection of Chondroitinase ABC activity

A qualitative assay for the detection of enzyme chondroitinase ABC activity was conducted [modified and adapted from (C. Rider and Hounsell E. F. 1998)]. A set of three tubes were prepared, with one containing 1 ml of growth media representing the negative control. A second tube contained standard chondroitin sulfate B (CS-B) solution (2 mg/ml) (from bovine mucosa, Sigma-Aldrich Co., Poole, U.K.) and 100 µl of enzyme chondroitinase ABC (2 U/ml) that represented the treated control or experimental tube. The third tube contained CS-B solution (2 mg/ml) with 100µl of

enzyme buffer representing the untreated control or positive control. CS-B solution for both the second and third tubes was prepared in growth media. After addition of one drop of toluene to each of the above tubes, they were tightly closed and incubated overnight at 37^{o}C. The enzyme activity was detected by the digestion of CS-B. This was determined by adding 100 µl of 10% (w/v) cetylpyridium chloride solution (Sigma-Aldrich Co., Poole, U.K.) to all the tubes, and a white opalescent precipitate establishes the presence of intact GAG. Only the positive control observed the presence of intact CS-B-GAG chains by forming a visible flocculant precipitation. However, the absence of precipitation in experimental test tube indicated that the enzyme preparation actively degraded CS-B chains and therefore was unavailable for a precipitation reaction.

2.5 Quantification of Secreted and Cellular IFN-γ by ELISA

A sandwich ELISA (indirect method) was performed for secreted and cellular IFN-γ, as per the manufacture's protocol of the OPTEIA kit (Pharmingen Inc, San Diego, CA).

2.6 Cell Viability Assay

50 µl of cell suspension was added to 20 µl of 0.1% Nigrosin stain solution (Sigma-Aldrich Co., Poole, U.K.) and mixed thoroughly. 20 µl of this mixture was applied to an Improved Neubauer Hemocytometer slide and cells were counted for viability under 40x magnification of light microscope. Non-viable cells were completely stained purple-blue due to penetration of dye whereas the viable cells remained unstained. The percentage cell viability was determined by comparing the number of unstained viable cells to the number of stained cells.

2.7 Preparation of Cell Lysates for SDS-PAGE

Adherent cells in the 6-well culture plate (Orange Scientific, B-1420 Braine-I' Alleud, Belgium) were treated with 500µl cell dissociation buffer. The contents of the well were collected in eppendorf tubes and spun at 1500 rpm for 5 minutes. To the cell pellet, 100µl of hot TBS- 1% SDS buffer and 5 x Laemmli's SDS sample buffer (4 ml distilled water, 1 ml of 0.5 M Tris-HCl buffer, pH 6.8, 0.8 ml glycerol, 1.6 ml of 10%

SDS, 0.4ml 2-mercaptoethanol, 0.2 ml of 5% bromophenol blue) was added. The samples were boiled for 5 minutes to lyse the cells and denature the proteins, then briefly cooled on ice. Further, to maximise the detection and identification of phosphorylated STAT-4, 1mM of activated pervanadate (PV) solution, 0.1% cocktail of Protease inhibitors (Sigma-Aldrich Co., Poole, U.K.) and Phosphatase inhibitors (diluted 1:100) (Sigma-Aldrich Co., Poole, U.K.) were added to the cell lysate. These additions protect the integrity of phosphoproteins for detection. The PV solution was prepared immediately prior to use by mixing 1M H_2O_2 and 1M sodium vanadate solution (both Sigma-Aldrich Co., Poole, U.K.) in a 1:1 ratio to produce 0.5M pervanadate solution which at 500x dilution with cell lysate resulted in 1mM final concentration. The samples were then stored at -20^{o}C. Where appropriate, the protein content in the cell lysate samples was determined using Pierce micro BCA protein assay following the manufacturer's instructions (see Section 2.8.2). Prior to use for SDS-PAGE (Section 2.9.1), the samples were thawed on ice, then boiled for 5 minutes, and centrifuged for 1 minute at maximum speed in a table-top centrifuge (1000 x g). Samples were run at an equal protein loading on 10% SDS-PAGE gels and analysed by western blotting.

2.8 Protein Assays

Where appropriate, the protein content in the samples was determined by either using a Coomassie Blue assay or a Microprotein assay.

2.8.1 Coomassie Blue Assays

The concentration of protein in a sample was measured in a 96 well microtitre plate (Dynatech Laboratories Inc.) using Coomassie Blue assay by absorbance at 595nm (Section 2.1.1). 20 µl of sample was mixed with 200 µl of Coomassie Blue reagent (Pierce Coomassie Plus Protein Assay reagent, Thermo Fisher Scientific, Rockford, USA). Absorbance at 595nm was measured against blank control (20 µl double distilled H_2O and 200 µl Coomassie Blue reagent). The average of three absorbance readings per sample was calculated and read against a calibration curve of a known bovine serum albumin (BSA) with concentrations from 0-1.5 mg/ml at intervals of 0.3. Standard BSA (Sigma-Aldrich Co., Poole, U.K.) solution was prepared in 0.25M ammonium bicarbonate buffer, pH 8.

2.8.2 Microprotein Assays

Protein content of samples was estimated using the Pierce micro BCA and was carried out according to manufacturer's instructions (Thermo Fisher Scientific, Rockford, USA). This method was used for some of the samples made up in SDS sample buffer.

2.9 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

2.9.1 SDS-PAGE

SDS-PAGE was carried out as previously described (Laemmli, 1970) using a Bio-Rad minigel apparatus. The apparatus was used to cast a 10% polyacrylamide separating gel by mixing 2.95 ml deionised H_2O, 1.9 ml separating buffer (1.5 M Tris-HCl buffer, pH 8.8), 2.5 ml of 30% (w/v) acrylamide (BDH chemicals, UK), and 75 µl of 10 % (w/v) SDS with 75 µl of 10% ammonium persulphate (APS) and 3 µl TEMED [tetramethylethylenediamine] (Sigma-Aldrich Co., Poole, U.K.) to allow polymerisation. This was followed by addition of 4% stacking gel solution prepared by mixing 2.9 ml deionised H_2O, 950 µl of 30% (w/v) acrylamide, 1.25 ml stacking buffer (0.5 M Tris-HCl buffer, pH 6.8), 31.5 µl of 10% APS and 7.5 µl TEMED. 10 µl/well of samples obtained from the above extraction method (Section 2.7) were loaded into the gel, along with 5 µl of prestained protein standard (SeeBlue Plus2 Pre-Stained Standard, range 4-250 kDa, Invitrogen, Paisley, UK). The casting apparatus with gels was placed in an electrophoresis chamber unit (Bio-Rad Laboratories, UK), filled with running buffer [25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS and pH 8.3]. Gels were elecrophoresed at a constant voltage of 200V in running buffer until the dye-bromophenol blue in the samples reached end of the gels.

2.9.2 Coomassie Blue Staining of SDS-Polyacrylamide Gels

To detect protein separation after SDS-PAGE, gels were stained with Coomassie blue solution [0.25% (w/v) Coomassie Brilliant blue R250 (Sigma-Aldrich Co., Poole, U.K.), 50% (v/v) Methanol, 10% (v/v) Acetic acid] for 15 minutes. Subsequently, gels

were destained with destaining solution [45% (v/v) Methanol, 5% (v/v) Acetic acid in deionised H_2O] until the protein bands were clearly visible against the background.

2.9.3 Western Blotting

Proteins resolved on SDS-polyacrylamide gel were transferred onto a nitrocellulose membrane of 0.45µm of pore size (Schleicher & Schwell, Whatman plc, Maidstone, Kent, UK) with a wet blotting technique (modified from (Towbin *Et. al.,* 1979) using the Bio-Rad minigel system (Bio-Rad Laboratories, UK). The gel-nitrocellulose membrane was assembled between two sheets of Whatman filter paper (3mm) and transfer pressure pads. This was then placed in a blotting tank containing chilled blotting buffer [25 mM Tris, 192 mM Glycine, 1% (w/v) SDS with 20% (v/v) Methanol]. Nitrocellulose membrane and Whatman papers were pre-soaked in blotting buffer for 15 minutes prior to blotting. Protein was transferred onto membrane by electrophoresis at a constant voltage of 100 V for 1hour. Following the transfer, gels were stained with Coomassie blue solution (Section 2.9.2). Alternatively, after transfer of proteins by electrophoresis, the nitrocellulose membrane was stained with Ponceau staining solution [0.5% (w/v) Ponceau Red (Sigma-Aldrich Co., Poole, U.K.) in 3% (v/v) acetic acid] to determine successful protein transfer onto the membrane and confirms the effectiveness of blotting procedure. Blots were washed three times in 1x TBS buffer (136mM NaCl, 19.8mM Tris base, diluted 1/10, pH 7.6) with 10 minutes per wash, dried, and preserved between Whatman papers until further used for immunodevelopment.

2.9.4 Immunodevelopment of Western Blots

Following western blotting, various cellular proteins, such as tyrosine phosphorylated STAT-4, serine phosphorylated STAT-4, unphosphorylated STAT-4, T-bet, and β-actin (loading control) were detected using a specific primary antibody for each of these proteins. Blotted membranes were washed three times in TBS with 10 minutes per wash then incubated for 30 minutes in blocking solution (2% marvel, 0.1% Tween-20 in 1 x TBS buffer) at R.T. Following this, the blots were incubated overnight at 4^{o}C with specific primary antibody diluted in blocking solution (see Table 2.2 for details of primary antibodies and their dilutions used). On the following day, blots were washed three times for 10 minutes each in TBS-T (0.1% Tween-20 in 1 x TBS buffer). The

blots were incubated in secondary antibody conjugated to horseradish peroxidase (HRP) enzyme [usually at a dilution of 1:1000 for polyclonal goat anti-mouse IgG (Dako, Denmark) and 1:5000 for polyclonal goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., USA)] in blocking solution for 1 hour at room temperature for chemiluminescent reaction. Following this, the blots were washed for three times with TBS buffer for 5 minutes each time. Chemiluminescent substrate (Pierce ECL, Thermo fisher Scientific, Rockford, USA) was applied to the blots for 5 minutes, according to the manufacturer's protocol. Blots were covered with cling-film and exposed to an appropriately sized film (Hyper-film, Amersham Biosciences Plc., UK) for some amount of time as required (varying from a few minutes to 1 hour), depending on the strength of chemiluminescence. Following this exposure, the film was placed in developing solution for 1-2 mins, rinsed with water and placed in fixer for 1-2 mins. The immunoreaction between the protein of interest and antibodies was visualised as bands, the intensity of which was measured using software – Image J. Densitometric analysis of bands on western blots was performed using blots from three independent experiments. However, densitometric analysis should only be viewed as semi-quantitative due to restrictions in sample numbers and is dependent upon the signal strength of chemiluminescence substrate and exposure time.

Table 2.2: Details of primary antibodies used for immunodevelopment of western blots

Primary Antibodies	**Dilution used**	**Company Details**
Mouse Monoclonal anti-T-bet (4B10)	1:200	Santa Cruz Biotechnology, C.A., USA
Rabbit polyclonal anti-phosphorylated (Y693)-STAT-4	1:200	Zymed Laboratories, CA, USA
Rabbit polyclonal anti-STAT-4 (C-20)	1:800	Santa Cruz Biotechnology, C.A., USA
Mouse monoclonal anti-β-actin (AC-15)	1:1000	(Sigma-Aldrich Co., Poole, U.K.)
Horseradish peroxidise conjugated Goat polyclonal anti-mouse IgG	1:1000	Dako, Denmark
Horseradish peroxidise conjugated Goat polyclonal anti-rabbit IgG	1:5000	Jackson ImmunoResearch Laboratories, Inc., PA, USA

Rabbit polyclonal anti-phosphorylated (S721)-STAT-4	1:200	Santa Cruz Biotechnology, C.A., USA

2.9.5 Stripping and Reprobing of Western Blots

Where appropriate, blots were stripped and reprobed for different proteins. For this, membranes were washed three times for 10 minutes each in TBS buffer, followed by incubation in Glycine-SDS buffer (25 mM Glycine buffer, pH 2.0, 1% SDS) for 30 minutes at room temperature on a rotating platform. The blots were washed three times for 10 minutes each in TBS-T buffer and incubated in a secondary antibody conjugated with HRP for 30 minutes at room temperature. Blots were developed on a film by applying chemiluminescent substrate. This step was optional as it detected the stripping efficiency and to check the removal of original signal. If this step was performed, the blot was washed three times in TBS-T for 5 minutes per wash, followed by incubation with specific primary antibody for detection of another protein.

2.10 Plasmids

pFL-IFN reporter plasmid (7.6kb) used in the transfection experiments is a -777bp to + 48bp murine IFN-γ promoter-driven luciferase construct, D3575 (-777), which was received as a kind gift from Dr. Howard Young (Laboratory of Experimental Immunology, NCI-Frederick, MD). Originally, this plasmid was constructed by Dr. Ken Murphy (Washington University, St. Louis) by cloning the IFN-γ promoter fragment from -777bp to + 48bp relative to the transcription start site (TSS) of the murine IFN-γ gene in pBS-LUC (~6.8kb), a firefly luciferase-based backbone vector with ampicillin resistance gene (Amp^r). pBS-LUC vector plasmid was generated as a high-copy-number, luciferase reporter plasmid by cloning 3.8kb *Pst*I-*Nde*I fragment from PXP-2 into *Sac*I-*Kpn*I sites of pBSII.SK- [2958bp, Bluescript II (SK-)] plasmids. pBS-LUC plasmid has also been previously used for creating variously-sized IL-2 (IL2-LUC) and IL-4 (IL4-LUC) promoter reporter vectors for tranfection in EL-4, T cell lines, as described in (Szabo *Et. al.*, 1993) suggesting that the vector could be successfully transfected for expression in mammalian cells.

To prepare an amount of DNA sufficient for transfection experiements, pFL-IFN reporter plasmid, once received was transformed into Library Efficiency DH5α *E.coli*

cells (Invitrogen) and a single colony of cells was selected from Luria-Bertani (LB) agar plate containing ampicillin. The plasmid DNA was extracted and purified by DNA mini-prep (QIAprep Spin Miniprep Kit, Qiagen Inc, Valencia, CA, USA), followed by DNA maxi-prep (EndoFree Plasmid Maxi Kit, Qiagen), according to manufacturer's protocol. DNA was quantified by a SmartSpec 3000, Spectrophotometer (Bio-Rad laboratories, Hercules, CA, USA) and absorbance readings at 260/280nm were calculated as per program settings in the instruction manual. Further, the construct was verified by sequencing performed by The Sequencing Service (College of Life Sciences, University of Dundee, Scotland) using standard primers, M13 Fwd (-20) and M13 Rev.

Since the experimental vector, pFL-IFN, was a firefly-based luciferase vector, a *Renilla* luciferase plasmid, pRL-TK (Promega, Southampton, UK), was used together to co-transfect KY-1 cells. Co-transfections with pRL-TK vector served as an internal control to normalise transfection efficiencies. Internal control reporter activity reflects the amount of DNA transfected into cells and their capability to express proteins. Therefore, normalisation of experimental vector with respect to co-transfected control vector considers transfection variability such as cell plating and transfection efficiency from one experimental or sample well to other. Thus, vector normalisation is generally recommended in for transient transfection of cells to control data variability arising from difference in transfection efficiency per sample well (Schagat *Et. al.*, 2007). pRL-TK is a commercially available, 4kb vector with the herpes simplex virus thymidine kinase promoter (HSV-TK) placed upstream of renilla luciferase gene. In the co-transfections, it is important to consider that a stronger promoter in the control vector could mean *trans* effects on experimental promoter activity. Therefore, HSV-TK, which is considered as a moderate promoter for the constitutive expression of renilla luciferase reporter gene in transfected mammalian cells was selected. Further, to ensure independent experimental and control reporter gene expressions, preliminary co-transfection experiments were performed to optimise the amount of total DNA and the ratios of control to experimental reporter vectors used. All transfections were internally controlled by pRL-TK reporter vector that is expressed under moderate but constitutive promoter, so it serves as internal control to normalise transfection efficiency.

2.11 Transient Transfections and Luciferase Analysis

2.11.1 Transient Transfection of KY-1 Cells

NK cells are one of the difficult cells to transfect. To our knowledge, transient transfection of KY-1 cells has not been previously reported. Therefore, the first attempt to transfect this cell line was using the Amaxa nucleofection technology ™ [Lonza (Amaxa), Basel, Switzerland] reported to be effective for NK cells (Maasho *Et. al.*, 2004). This contemporary nucleofection method was found to be highly efficient compared to other conventional methods of gene transfections, such as GeneJuice transfection reagent (Novagen, Germany) and lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), that were carried out at the same time.

For transfections, KY-1 cells were grown in T75 flasks to scale-up cell numbers. Once the cells in the flasks reached 80-85% confluence, they were used for transfection. Cells in highly proliferative stage (log-phase) were used, counted and cell viability was determined (Section 2.6) to be >90% and therefore optimal conditions for transfections. Transfections were performed as per the manufacturer's protocol for the various methods used. Efficiency of the method was validated by the expression of either pEGFP plasmid (4.7kb vector encodes enhanced green fluorescent protein, Clontech Labs., CA, USA) or pmaxGFP® [3486bp plasmid encodes green fluorescent protein, Lonza (Amaxa), Basel, Switzerland] as the marker in a single plasmid transfection as well as in co-transfections with the pRL-TK vector. Transfections using GeneJuice and Lipofectamine 2000 resulted in poor efficiency in comparison to the nucleofection approach. Rather, post-nucleofection yielded 15-20% transfection efficiency and cellular confluency of ~40-60% in wells using 'V' solution kit [Lonza (Amaxa), Basel, Switzerland]. Initial co-transfections performed using Amaxa nucleofection technology, included pmaxGFP®, and pRL-TK vectors in the proportion of 1 (control) to 5 (experimental) with the total of 10µg vector DNA added to 2.5 x 10^6 cells. Dupicate wells per condition were prepared with appropriate controls, including negative controls (cells nucleofected without DNA) and non-transfected (NT) wells.

Further, transfection conditions were optimised for co-transfections of KY-1 cells with pFL-IFN (murine IFN-γ reporter construct, experimental) and pRL-TK (control) vectors to determine the amount of total vector DNA and the ratio of reporter vectors

used. Briefly, KY-1 cells in log-phase growth were harvested and nucleofected with plasmids, pRL-TK (1.65µg) and pFL-IFN (8.35µg) in the proportion of 1 to 5. Each cuvette contained 100 µl of 4 x 10^6 cell suspension mixed with total 10µg plasmid DNA in solution 'V' and nucleofected using Nucleofector™ II device (Lonza (Amaxa), Basel, Switzerland) set for program T-020 (Manufacturer's handbook). Nucleofected cells were immediately transferred into the wells of 6-well plates containing 1ml pre-warmed growth media with IL-2 and incubated at 37°C, 5% CO_2. After a rest period of 5 hours, culture media was replaced to remove cell debris produced on nucleofection, with fresh growth media containing IL-2 and continued for overnight incubation. Next day, transfected wells with ~40-50% confluency were gently washed with fresh growth media without IL-2 and replaced with the same. At this time, cells were treated with TGF-β1 (10ng/ml) for 30 mins followed by stimulation with IL-12 (10ng/ml) alone or IL-12 plus IL-18 (100ng/ml) for 3 hours. Duplicate wells per cytokine treatment group including untreated group (no cytokines) were prepared. Appropriate controls such as negative controls (nucleofected cells without DNA) and non-transfected (NT) wells were treated with the same procedure as experimental wells. After this cytokine stimulation of transfected cells, supernatant culture media was collected to determine the endogenous IFN-γ levels by ELISA (Section 2.5). Cells were lysed and extracted for luciferase activity (Section 2.11.2).

2.11.2 Luciferase Assays by Dual-Luciferase® Reporter Assay System

The Dual-Luciferase® Reporter Assay System (DLR®) (Promega, Southampton, UK) was used, which allows for the detection and measurement of both firefly and *Renilla* luciferase activities from a single sample of cell lysate, as per the manufacturer's protocol. Briefly, after specific cytokine stimulations, transfected KY-1 cells were washed twice in PBS. 250 µl of passive lysis buffer (PLB) was added and plate was incubated for 20 mins at R.T. on a rotating platform. To ensure complete cell lysis, the contents of the well were mixed by active pipetting. Two aliquots of 100µl each, per cell lysate sample were prepared. On addition of each 100µl of sample aliquot to 100µl luciferase reagent (LAR II), both firefly luciferase, followed by renilla luciferase activities were measured sequentially using a luminometer (Turner Biosystems luminometer model TD 20/20; Promega, Southampton, UK). The instrument was set for 2-second pre-measurement delay followed by 12-seconds measurement period, which means that on mixing the sample lysate with LAR II, the luciferase activity was

measured as light emission over a period of 12 seconds. The Luminometer was also programmed to take three readings per sample that were averaged. The background signal due to the instrument, sample tubes and luciferase reagents was determined by preparing a cell lysate from non-transfected (NT) control cells and same steps as samples to measure luminescence was performed. This background measurement was subtracted from each sample reading. Three independent transfection experiments were performed with each experiment containing duplicate wells per cytokine treatment group.

Since all transfections were internally controlled by co-transfection with the pRL-TK vector, for each transfected well, firefly luciferase activity (experimental) followed by the activity of renilla luciferase (control) were determined in Relative Light Units (RLU) and their ratio (F/R) was obtained to normalise the sample values. After normalisation of values to the control (pRL-TK expression), pFL-IFN expression under various cytokine treatment was compared to its baseline expression in untreated (no cytokine) wells, for which the ratio (F/R) was taken as '1'. This determines the normalised fold change in activity per cytokine treatment and also gives the relative difference in activity between different treatment groups.

2.12 Statistical Analysis

Data were presented as mean ± standard deviation (SD), or ± standard error mean (SEM) as appropriate. Comparative data were analysed using unpaired Student's *t* test and one-way ANOVA with post hoc test. As appropriate, for some of the data (Chapter 4), statistical significance was determined by the non-parametric Mann-Whitney's test and Kruskal-Wallis test for group comparison. A p value of ≤ 0.05 was considered as statistically significant. All statistical analysis was performed using SPSS (versions 14.0 and 16.0 software).

CHAPTER 3

INVESTIGATION OF BINDING OF INTERLEUKINS 11, 18 AND 22 TO HEPARIN AND HEPARAN SULPHATE

3.1 Introduction

Interleukins, IL-11, IL-18 and IL-22, share close structural relationships to their known heparin-binding family members. Thus, it was hypothesised that heparin binding is a common feature among the interleukins within their respective family. The goal of this chapter is to investigate whether these three interleukins, IL-11, IL-18 and IL-22 from different cytokine families can bind to heparin. Moreover, the current investigation stems from the knowledge that the selected interleukins displayed potential heparin-binding domains (HBDs) which corresponds to the cluster of basic residues in their primary structure. In all the experimental and theoretical studies conducted here, heparin has been used as a representative compound for HS. Heparin being a pharmaceutical product is readily available in bulk as a highly sulphated analogue of HS. Because of this commercial availability of heparin, it is most commonly employed for protein-HS interaction studies.

The current investigation followed a systematic approach to ensure a comprehensive structural and biochemical study. For this, a combination of strategies such as predictive docking calculations and biochemical method like ELISA were employed to investigate the possible binding of IL-11, 18 and 22 to heparin. Initially, the amino acid sequences of the chosen set of interleukins were examined to identify the potential clusters of basic residues in heparin binding. Thus, the number and distribution of basic amino acids was determined from the primary structures of IL-11, IL-18 and IL-22. Subsequently, the clustering of basic amino acids that could potentially form a binding site for heparin chains on a three-dimensional protein structure was inspected. This was further developed by employing docking calculations based on the high-resolution structures of the interleukins with those of heparin pentamers and endecamers to predict the possibility of heparin binding. This predictive approach was based on minimum interaction energy level calculations. Finally, the experimental validation of binding of interleukins 11, 18 and 22 to heparin was performed using a specialised heparin-binding ELISA technique. This technique makes use of an immobilised heparin-BSA complex coated onto ELISA plate wells.

3.2 Results

3.2.1 Examination of the Amino Acid Sequences of IL-11, 18 and 22

An initial step in determining that a given protein might interact with heparin/HS GAG is to screen its amino acid or primary sequence. Screening of primary sequences can reveal any clusters or groups of basic residues within a short peptide region. It has been well-established that the basic residues, arginine and lysine largely contribute towards GAG binding (Caldwell *Et. al.*, 1996; Fromm *Et. al.*, 1997; Hileman *Et. al.*, 1998). Hence, to consider the interleukins 11, 18 and 22 as likely candidates for heparin-binding studies, the overall number and clusters of basic residues within the protein at the sequence level was examined first.

As seen in Figure 3.1, the primary sequences of mature proteins, IL-11, -18, and -22 contained high number of basic residues, arginine (R) and lysine (K) (colour coded). IL-11 contains 20 basic residues (11.2%) out of total 178 amino acids; IL-18 has 22 basic residues out of total 157 amino acids (14%), and lastly IL-22 has 18 basic residues (12.3%) out of total 146 amino acids. These numbers were comparable to other known heparin-binding cytokines, such as IL-6, which contained 23 basic residues (12.6%) in total of 182 amino acids, and GDNF with 25 basic residues (18.5%) out of total 135 amino acids.

Nevertheless, besides the high number of basic residues, arrangement of these residues in the primary sequence of proteins constitute sequence-motifs for heparin binding. A typical heparin-binding site (HBS) generally, consisted 4-6 basic residues in a single cluster within the linear arrangement of amino acids, or on the surface of a higher order of a structurally folded protein (Hileman *Et. al.*, 1998). As seen in Figure 3.1 (yellow highlight), primary structure of IL-11 highlighted the presence of a basic sequence-motif (^{111}RLDRLLRR^{118}) that closely resembled Cardin-Weintraub motif. Additionally, though IL-18 (^{129}KERDLFKLILKK^{140}) and IL-22 (^{114}KLKDTVKK^{121}) also exhibited presence of a cluster with about 4-6 basic residues near their carboxyl-terminus (highlighted in yellow), these basic clusters did not exactly match any known consensus sequence-motifs. However, these clusters in IL-18 and IL-22 may represent continuous heparin-binding sites. Particularly these carboxyl-terminal basic peptides are part of most HBS/HBDs of cytokines (Kuschert *Et. al.*, 1998; Lortat-Jacob *Et. al.*, 2002). Therefore, this primary sequence analysis was a preliminary test. It can be

particularly useful in predicting and correlating basic sequences or clusters to HBD in the three-dimensional structure of proteins.

Besides these clustered basic residues in the linear arrangement, a good number of basic residues were also evenly distributed through the entire primary sequences of IL-11, 18 and 22 proteins. This can lead to discontinuous-centres for heparin binding in which the single and dispersed basic amino acids not close in the linear sequences of IL-11, 18 and 22, come together to form clusters on higher order of their structural arrangements. Moreover, as explained earlier in Section 1.4.3 that the interaction with heparin chains is also dependent on the accessibility of these basic residues and their side chains on the surface of a three-dimensional protein structure. Hence, this further necessitates inspection of three-dimensional structures of IL-11, IL-18 and IL-22.

IL-11

1 PGPPPGPPRVSPDPRAELDSTVLLTRSLLADTRQLAAQLRDKFPADGDHN 50
51 LDSLPTLAMSAGALGALQLPGVLTRLRADLLSYLRHVQWLRRAGGSSLKT100
101 LEPELGTLQA111RLDRLLRR118LQLLMSRLALPQPPPDPPAPPLAPPSSAWGGI 150
151 RAALAILGGLHLTLDWAVRGLLLLKTRL178

IL-18

1 YFGKLESKLSVIRNLNDQVLFIDQGNRPLFEDMTDSDCRDNAPRTIFIIS 50
51 MYKDSQPRGMAVTISVKCEKISTLSCENKIISFKEMNPPDNIKDTKSDII 100
101 FFQRSVPGHDNKMQFESSSYEGYFLACE129KERDLFKLILKK140EDELGDRSIM 150
151 FTVQNED157

IL-22

1 APISSHCRLDKSNFQQPYITNRTFMLAKEASLADNNTDVRLIGEKLFHGV 50
51 SMSERCYLMKQVLNFTLEEVLFPQSDRFQPYMQEVVPFLARLSNRLSTCH 100
101 IEGDDLHIQRNVQ114KLKDTVKK121LGESGEIKAIGELDLLFMSLRNACI 146

Figure 3.1: **Primary sequences of the ILs-11, -18, -22 showing basic amino acids in putative heparin/ HS binding:** The amino acid sequences of mature, secreted forms of human (Swiss-Prot accession numbers) IL-11 (P20809), IL-18 (Q14116) and IL-22 (Q9GZX6) are shown with all amino acid residues represented by one letter code. The basic amino acids are shown as coloured letters, arginine = **R** and lysine = **K**. Primary sequences with four or more basic residues in proximity are highlighted in yellow. Superscript numbers refer to amino acid numbering in the secreted protein without signal peptide.

3.2.2 Structural Studies and Molecular Docking Calculations with IL-11, 18 and 22

This structural study involved inspection of a high-resolution, three-dimensional structure of a selected interleukin to gain more insight into its surface features, particularly positively-charged patches/clusters that can possibly interact with heparin. Firstly, structural studies of interleukins, IL-11, IL-18 and IL-22 enabled confirmation of the presence of basic clusters that appeared significant from the primary sequence studies (Figure 3.1), onto the surface of the three-dimensional structures of the proteins. Secondly, it also helped to identify possible discontinuous HBSs formed by isolated basic residues, distant in sequence but which form into positively-charged clusters on tertiary and quaternary structural folding. Finally, for the protein structures that were experimentally determined and available in the protein data bank (PDB), such as for IL-18 (1J0S) and IL-22 (1M4R), molecular docking studies were applied to predict possible heparin binding and to locate HBS on the surface of these interleukins. Molecular docking calculations were performed on our behalf by a collaborator, Dr. Barbara Mulloy (National Institute for Biological Standards and Control, NIBSC). These studies were carried out as per the method (described in Forster & Mulloy, 2006; Mulloy & Forster, 2008). However, for IL-11, an experimentally-resolved structure (4MHL) was very recently published (T. L. Putoczki *Et. al.*, 2014), therefore the docking calculations were not conducted for this protein at the time of this study.

Below is the brief description of the molecular docking method employed in the current study. Molecular docking also referred to as docking calculations, is a more systematic approach in identifying HBDs on a protein-surface. It also determines free binding energy values, called energy scores that calculate binding affinity between a ligand and a protein. Several published reports have used different software options for this purpose (Bitomsky W., 1999); however, the research work conducted in this study was based on using Autodock 2.4 version of software (Oxford Molecular Inc.) (G. M. Morris *Et. al.*, 1996). The docking protocol starts with screening the entire surface of a three-dimensional protein structure for the presence of basic clusters that may offer a suitable binding fit to negatively-charged groups on heparin chains. This is followed by docking several well-defined, semi-flexible heparin oligosaccharide ligands onto the protein structures. Because of the structural diversity in heparin chains, the highly charged-groups and their conformational flexibility can complicate the application of

this technique. Hence, the docking protocol uses a generic heparin model ligand based on the NMR structure-1HPN. The structural details of 1HPN has been briefly described earlier (Section 1.3.3). This model ligand is available in the PDB (Protein Data Bank) files and has been used to predict binding sites on the surfaces of many proteins with remarkable success (Mulloy *Et. al.*, 1993). High-resolution structures of interleukins IL-18 and IL-22 used in this study were also available in the PDB and were determined experimentally by either crystallography or NMR.

The most mutually favourable binding mode for the heparin-protein complex that yields the lowest inter-molecular interaction energy (kcal/mol) was determined. Complexes with predicted energies below -1000 kcal/mol were regarded as proteins having the capacity to bind heparin. In addition to predicting the location of heparin-binding sites on the target protein, the molecular docking can also determine the key basic residues involved in the interaction. However, the docking method does not provide details on the contribution of specific sulphate groups on the heparin ligands to the interaction. Moreover, this molecular docking method has also limitation in the case of proteins that require GAGs for oligomerisation. Therefore, it is important to know whether the monomer or higher oligomer of a protein is more appropriate for GAG binding. Since IL-22 exists in both monomeric and dimeric forms, docking calculations were applied to each form separately.

The docking protocol used in the current study has been very well validated using prototypical heparin-binding proteins such as FGF-1, FGF-2, and antithrombin III. It has also been successfully used for predicting heparin binding to various cytokines, chemokines and growth factors (Mulloy & Forster, 2000; Mulloy & Linhardt, 2001). However, the outcome of this molecular docking calculation is only predictive. This simulation method has been useful to illustrate and rationalize data from biochemical and experimental studies. In the current study, predictions from docking calculations in combination with heparin-binding ELISAs were employed to determine the affinity between heparin and the selected interleukins.

3.2.2.1 Structural Model of Human IL-11

At the time of this study, an experimentally derived, high-resolution structure of IL-11 was not available. Largely, various homology models of IL-11 were proposed using

other members of long-helical or IL-6 family of cytokines to understand structure-function relationship. Since, a group of basic residues within a short-peptide region was identified from the primary sequence data of IL-11 (Figure 3.1), it was decided to examine the proposed structural homology model of IL-11 to potentially reveal the location of these basic residues and hence a putative heparin-binding domain. The homology model of IL-11, constructed by Czupryn *Et. al.*, (1995a) provided structural details that were useful in analysing and understanding features in heparin binding. This proposed homology model, as shown in Figure 3.2A was constructed based on amino acid sequence homology and common secondary structural units (four-helix bundle topology), a characteristic feature within the family of long-chain, haematopoietic cytokines to which IL-11 belongs.

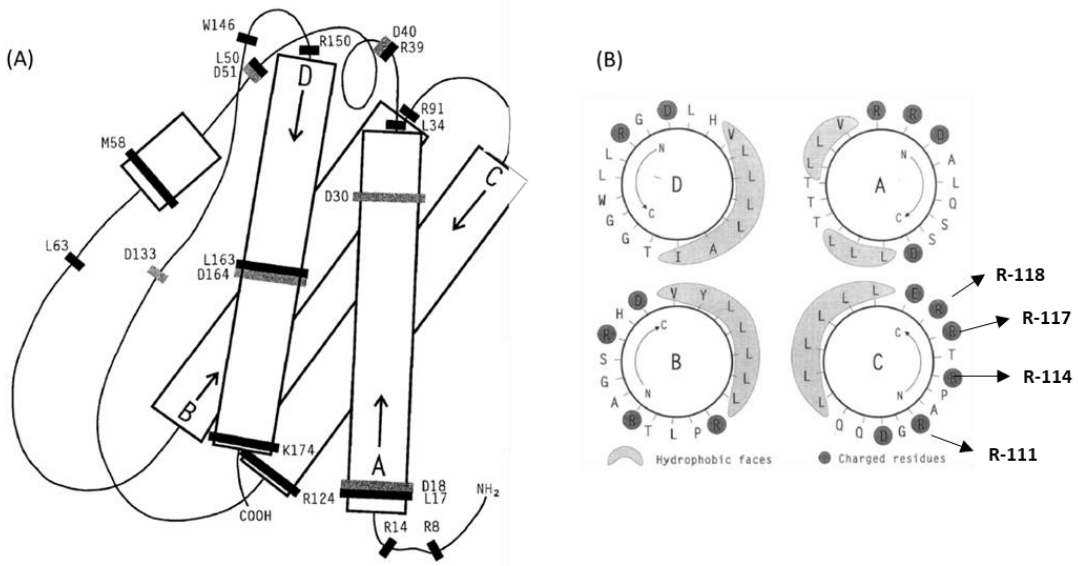


Figure 3.2 Proposed structural model of IL-11: Based on the sequence similarity of IL-11 with the members of long chain, α-helical cytokines, secondary structural elements were proposed that resulted in a typical four-α-helical bundle topology, characteristic of haematopoietic cytokines (M. J. Czupryn *Et. al.*, 1995a). A) Topological arrangement of four α-helices labelled A-D connected with two loop regions (loops AB, CD) are shown with the arrows indicating N- to C-terminal orientation. B) Top view of the four-α-helix bundle structure of IL-11 is shown. Hydrophobic residues facing the core of the protein structure are highlighted together in a crescent shape. Surface-exposed, charged residues are highlighted in circles for all helices. A notable group of Arg [R] residues (at positions 111, 114,117 and 118) are seen on the exposed surface of helix C. These basic residues could be postulated to be important surface contacts for interaction with heparin chains. This figure is adapted from (M. J. Czupryn *Et. al.*, 1995a).

As seen in Figure 3.2, IL-11 is proposed as an α-helical protein with four helices labelled A-D connected by loops. In Figure 3.2B, the hydrophobic residues are seen

lying at the interface formed by α-helices. However, an interesting feature observed in this structural model of IL-11 was an array of charged arginine (R) residues (Arg 111, 114, 117 and 118) that formed a part of the solvent-exposed surface of helix C. Indeed, these basic residues forming a cluster on helix C belonged to the basic sequence-motif (^{111}RLDRLLRR^{118}), identified in the primary sequence of IL-11 (Figure 3.1). Therefore, this basic peptide region identified from the primary sequence analysis was apparently a surface-exposed feature and could be postulated as important in the interaction with heparin chains. However, this observation could not be confirmed by the molecular docking strategy. This was because, application of docking calculations to the structural homology models would have introduced higher uncertainty in the docking protocol used and may result in an artefact (Mulloy & Forster, 2008).

Additionally, this proposed structural model for IL-11 and the observation regarding the surface-arginine residues are in good agreement with the recently reported crystal structure of IL-11 (T. L. Putoczki *Et. al.*, 2014) (Figure 3.3; PDB ID 4MHL). Accordingly, the crystal structure of IL-11 comprised of four core helices (Figure 3.3A, labelled A-D) with long loop regions between helix A-B and C-D. Moreover, structural analysis by Putowczki et. al. showed the presence of aforementioned basic residues (Arg 111, 114, 117 and 118) as a positively charged patch. This was seen, particularly on the surface formed by helices A and C, as revealed from the distribution of surface electrostatic potential in IL-11(Figure 3.3B). Interestingly, a comparative structural study between IL-11 and IL-6 by Putoczki et al highlighted the presence of an extended area of positive electrostatic potential in IL-11 as a distinguishing feature. Whilst, IL-6, a structural and functional counterpart of IL-11 within family, exhibited more concentrated patches of positive surface potential (T. L. Putoczki *Et. al.*, 2014).

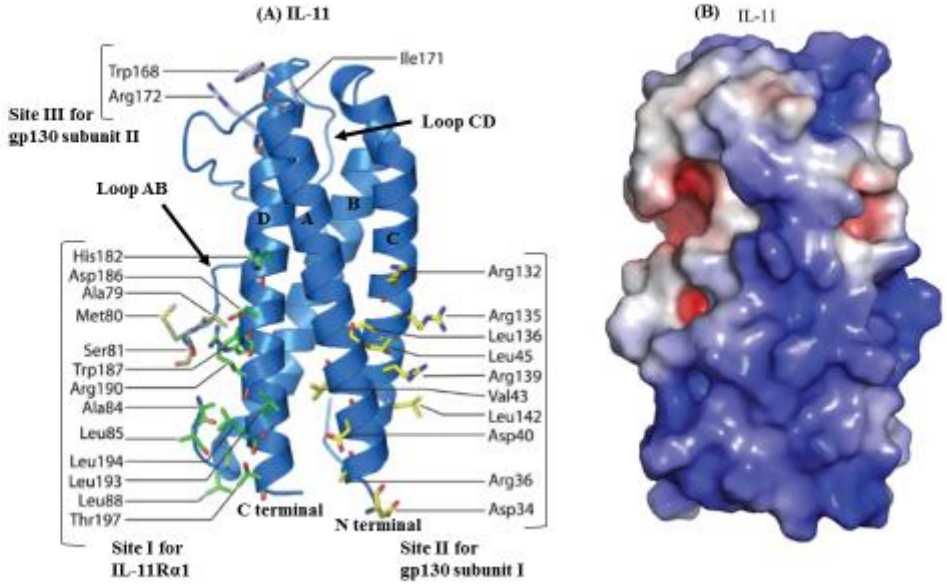


Figure 3.3 Crystal structure of human IL-11 (PDB Id 4MHL): (A) Schematic representation of the IL-11 structure as ribbons indicating four alpha helices (labelled A-D) and loop regions. Residue contacts in binding to IL-11Rα (Site I) and to subunits of gp130 (Site II, Site III) are shown in stick model. Arg residues on helix C form part of putative heparin-binding region on IL-11 as well as gp 130 receptor-binding site II. **(B) Surface representation of the IL-11 structure**. Negative (red) and positive electrostatic potential (blue) is shown for IL-11. The presence of Arg residues (132, 135 and 139) on helix C is seen forming a large area of positive potential between helices A and C, around Site II. The orientation of both structural representation (A) and (B) is same. Residue numbering is according to the full-length precursor sequence of IL-11. Figure adapted from (Putoczki *Et. al.*, 2014).

Since, receptor binding and heparin binding, both results from the involvement of surface elements, it is an important comparison in further understanding IL-11-heparin/HS interaction and its functional implications. The chemical modifications and site-directed mutagenesis experiments revealed the residues important for receptor-binding and the biological activity of IL-11(tacken 1999, (M. J. Czupryn *Et. al.*, 1995a; M. Czupryn *Et. al.*, 1995b). These residues have been grouped into three sites as shown in the crystal structure of IL-11 (T. L. Putoczki *Et. al.*, 2014), Figure 3.3A. Indeed, the comparison of receptor-binding residues with the predicted heparin-binding residues, Arg 111, 114, 117, and 118, from primary sequence data (Figure 3.1), showed overlapping at the interaction site (Site II) in IL-11 for signalling receptor subunit, gp130. Additionally, three of the arginine residues from the potential heparin binding site were observed to be important for IL-11 activity via receptor site II for gp130 subunit (Figure 3.3A). This observation indicated that the interaction of IL-11 with

heparin/HS would interfere with its binding to gp130 molecule and hence signalling. Therefore, based on the spatial distribution and/or orientation of basic residues on a three-dimensional structure of IL-11, they might be available for possible heparin binding. However, further biochemical study was used to determine whether IL-11 possesses heparin-binding property or not.

3.2.2.2 Examination of the Structure of Human IL-18 for Possible Heparin-binding Sites

An experimentally determined structure of IL-18 was available. As mentioned earlier, IL-18 exhibits the β-trefoil structural fold, a 'signature scaffold' present in all members of the FGF family that generally results in HSGAG-binding regions (Raman *Et. al.*, 2003). The available three-dimensional structure of IL-18 was inspected for the location of possible heparin-binding sites. As seen in Figure 3.4, a high-resolution, three-dimensional NMR structure of IL-18 (Kato *Et. al.*, 2003) was computed and the residues are shown colour-coded (red/blue) as per electrostatic potential. Accessible and exposed surfaces of the protein structure was inspected for the presence of basic clusters that can be available for interaction with heparin. As seen on the left-side of Figure 3.4 (yellow coloured residues), this structure possessed two-independent, positively-charged clusters formed by the basic residues close to the carboxyl-terminus. One of the clusters includes residues Lys-129 and Arg-131, while the other comprised of Lys139 and Lys-140 (they have been circled and coloured yellow in Figure 3.4). Unexpectedly, a cluster of basic residues that was identified within a short-peptide region from the primary sequence data of IL-18 (Figure 3.1), comprising of Lys 129, Arg 131, Lys 139 and Lys 140 from the β strands of β10-β11 was seen transformed into two distinct, putative heparin-binding sites on higher order of tertiary structural fold. Moreover, it should also be noted that these two basic clusters (Figure 3.4) were interspersed by the adjoining surface acidic residues, Glu 128, 130, 141, 143 and Asp 132, 142 that may repel possible ionic interactions with heparin chains.

Further examination of the structure of IL-18 revealed a third putative interaction site for heparin. This was a surface cavity formed by the loop regions of β-strands, β4-β5 and β7-β8 and consisted of basic residues, Lys-53 and Lys-93 with their protruding side chains (highlighted as yellow colour on right side of Figure 3.4). Moreover, at this site, the adjacent acidic residues were directed towards the centre core of the protein

structure and not surface-exposed, possibly enabling interaction with heparin. However, only two basic residues may not be enough to offer a stable binding-site with strong electrostatic forces to attract and hold interactions with heparin chains. Further, molecular docking and energy calculations will be useful in determining the feasibility of this basic cluster in forming a heparin-binding site. Overall, based on the observations from the three-dimensional structural study, the possibility of heparin binding to IL-18 remained low.

Nevertheless, it was significant to obtain more information on amino acids that formed a critical part of IL-18 structure. It was next sought to locate the residues involved in receptor binding on the surface of IL-18. This highlights whether the receptor-binding site spatially differs or overlaps with the above proposed heparin-binding sites in IL-18, suggesting possible structural or steric constraints for heparin/HS interactions. Site-directed mutagenesis, deletion, and charge reversal studies demonstrated Glu 6 and Lys 53 (coloured green in Figure 3.5) as critical amino acids for the entire IL-18 structure-function relationship from receptor-binding to signal transduction initiating a cellular response (Kim *Et. al.*, 2002). Acidic residues Asp 17, Asp 35 or Asp 132 (coloured aqua green in Figure 3.6A) on the surface of IL-18 were shown to be important in binding to its cognate receptor, IL-18Rα (Kato *Et. al.*, 2003). Other functionally important residues were Lys 79, Lys 84 or Asp 98 (coloured purple in Figure 3.6B), significant in binding to IL-18Rβ for ternary ligand-receptor complex formation (Kato *Et. al.*, 2003). Also, recent structural and mutational studies identified an important role for residues His 109 and Lys 112 in IL-18 binding to its with signalling receptor subunit, IL-18Rβ (Kato *Et. al.*, 2014). Further, comparing the receptor-binding residues (for both IL-18Rα and β) with the above proposed heparin binding residues indicated potential overlapping functions for a single, basic residue, Lys 53 (Figures 3.4, 3.5 and 3.6). Therefore, physiologically, this indicates minimal structural constraints in IL-18 for possible heparin binding.

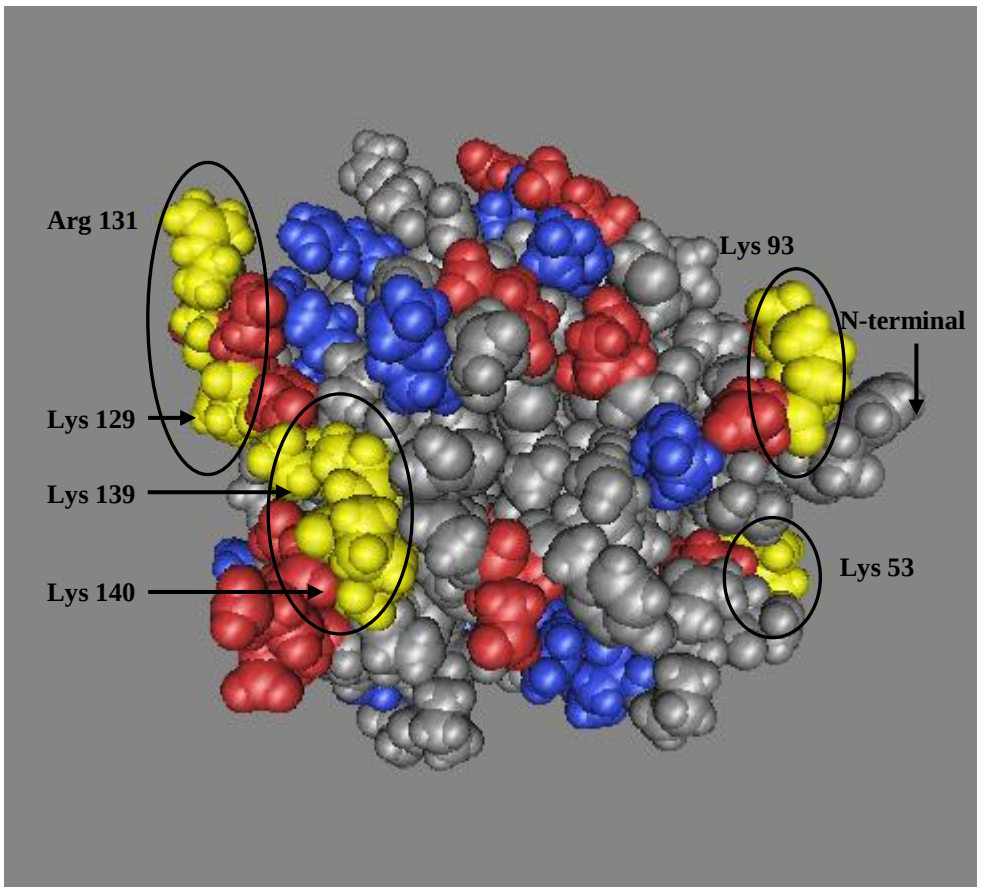


Figure 3.4: NMR structure of IL-18 (mmdbId: 25207) showing basic clusters involved in putative heparin-binding sites: The structure is available in PDB (1J0S). It is viewed and annotated using the Cn3D programme and represented in a space-filled model. The surface residues are colour-coded according to the electrostatic potential: basic residues are in blue, acidic in red. Basic amino acids predicted in heparin binding, either singly or in clusters, are coloured yellow, circled and labelled. The structure in this view is 180^{o} in orientation to the structure in ribbon style of Figure 3.7 across the central horizontal line of axis.

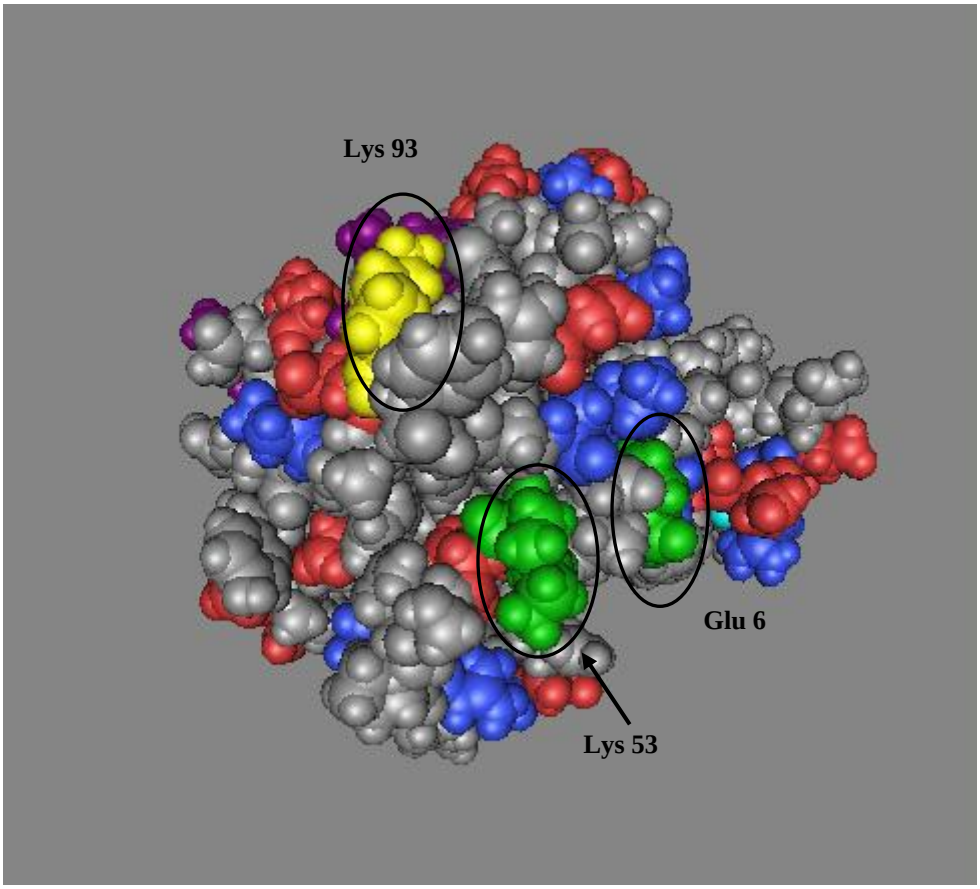


Figure 3.5: NMR structure of IL-18 (mmdbId: 25207) showing critical residues for the structure-function of IL-18: The structure is available in PDB (1J0S). It is viewed and annotated using the Cn3D programme and represented in a space- filled model. The surface residues are colour-coded according to electrostatic potential: basic residues are in blue, acidic in red, and residues involved in IL-18Rα binding are purple. Two critical amino acids, Lys-53 and Glu-6 required for structural-function integrity of IL-18, are coloured green, circled, and labelled. The structure in this view is 90^{o} in orientation to the left of the structure in Figure 3.4 across the central vertical line of axis.

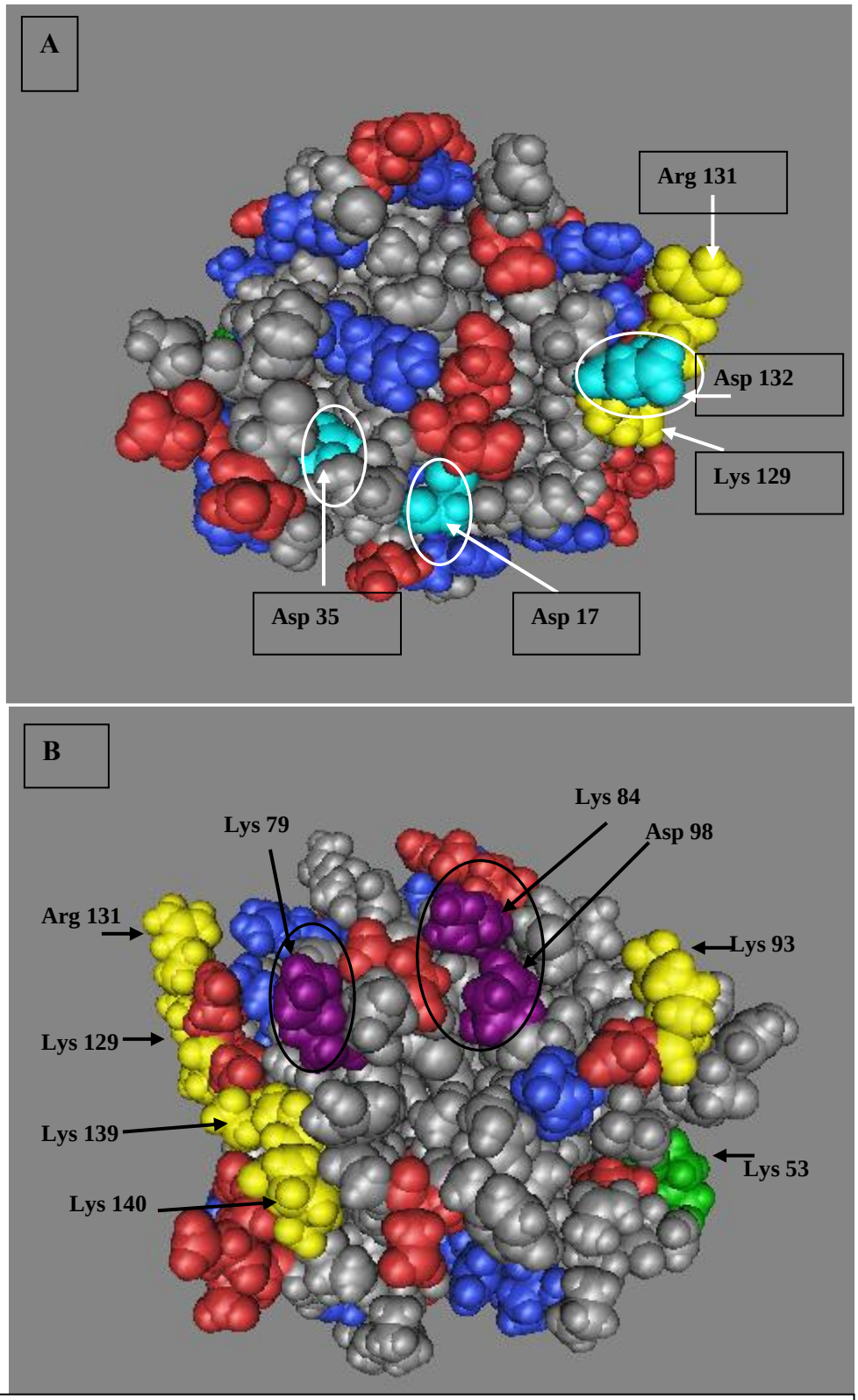


Figure 3.6: NMR structure of IL-18 (mmdbId: 25207) showing key residues involved in the IL-18-receptor (IL-18Rα / IL-18Rβ) binding: The structure is available in PDB (1J0S). It is viewed and annotated using the Cn3D programme and represented in a space-filled model. The surface residues are colour-coded according to electrostatic potential: basic residues are in blue, acidic in red and basic amino acids predicted in heparin binding either singly or in clusters are coloured yellow. **A)** Three key amino acids residues involved in IL-18Rα binding are coloured aqua green, circled, and labelled. The structure in this view is 90° in orientation to the right of the structure in Figure 3.4 across the central vertical line of axis. **B)** Three key amino acids residues involved in ternary complex formation (IL-18: IL-18Rα: IL-18Rβ) are coloured purple, circled, and labelled. The structure in this view is super-imposable in orientation to the structure in Figure 3.4.

3.2.2.3 Molecular Docking of IL-18 protein with Heparin endecamer ligand

To further ascertain the possible binding of heparin to IL-18, molecular docking studies were conducted. The NMR structure of IL-18 (mmdbId: 25207) was systematically docked on a heparin endecamer (mmdbId: 3448) using Autodock 2.4 to determine a model with the lowest interaction energy (Mulloy & Forster, 2008). Figure 3.7 shows the computational interaction between structural IL-18 and the heparin endecamer. This programme performed a complete survey of the surface of IL-18 with heparin chains and, hence could identify a putative heparin-binding site. Interestingly, with IL-18, residues Lys 53 and Lys 93 (Figure 3.7) facilitated favourable ionic interaction with the docked heparin chains. These two basic residues were situated in the region brought together by the loops connecting two pairs of anti-parallel β sheets (β4-β5 and β7-β8). Ten repeat docking calculations were performed. This molecular docking data also validated the proposed heparin-binding site that was observed on inspection of the three-dimensional structure of IL-18, (highlighted as yellow colour on right side of Figure 3.4).

The interaction energy of the complex was calculated as -200 kcal/mol. This value is much higher than -1000 kcal/mol, a threshold value. A value below this threshold is generally considered to indicate protein ability to bind heparin. Moreover, this energy value is relatively much higher when compared to the interaction energy values of -2200 kcal/mol and -3500 kcal/mol calculated for the experimentally verified, heparin-binding interactions of IL-12 and FGF-2 respectively with the pentameric heparin oligosaccharides (collaborator's personal communication). Thus, high intermolecular interaction energy predicted from molecular docking simulation suggested an unstable binding complex. Also, with only two basic residues, Lys 53 and Lys 93 involved at the possible heparin-binding site, this suggested that IL-18 was unlikely to bind heparin. Although molecular docking simulation was used as a predictive tool, further experimental evidence was needed to determine whether IL-18 has a measurable affinity for heparin.

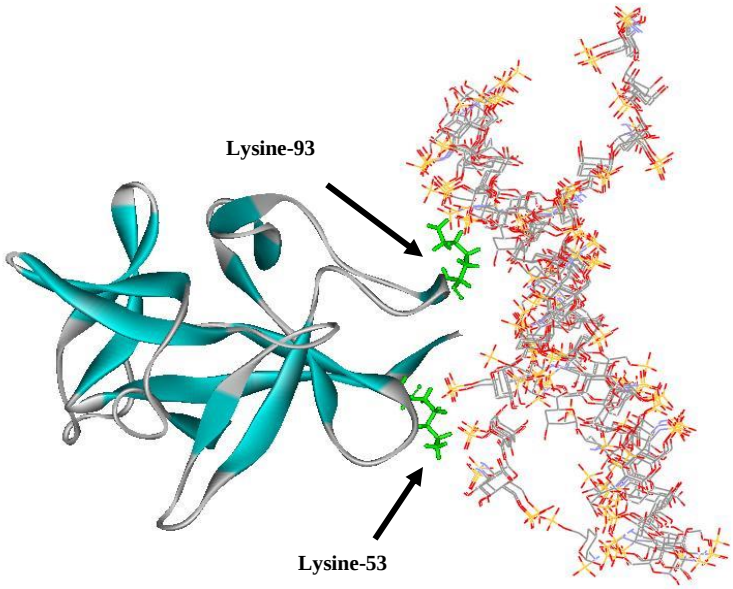


Figure 3.7: Interaction of the human IL-18 with a heparin endecamer: The β-trefoil structure of IL-18 has been represented by ribbons with β sheets coloured in aqua green. The NMR structure of IL-18 was complexed with the heparin endecamer and ten superimposable lowest energy complexes are shown. The interaction energy of IL-18-heparin complex was calculated and the basic residues (Lysine-53 and 93) identified as possible parts of heparin binding site, shown as green stick form. The docked heparin chains are shown as stick models with carbon-black, oxygen-red, nitrogen-blue and sulphur-yellow. The docking predictions were performed with Autodock version 2.4 (Mulloy & Forster, 2008).

3.2.2.4 Examination of Monomeric and Dimeric Structures of Human IL-22 for Possible Heparin-binding Sites

Like IL-18, IL-22 also has an experimentally determined crystal structure (Nagem *Et. al.*, 2002; T. Xu *Et. al.*, 2005). IL-22 exists in both monomeric (Figure 3.8) and dimeric forms (Figure 3.12). Because of this, IL-22 may possess two independent heparin-binding sites, one on each monomer or a single co-operative binding site on a dimer involving both the monomers. Hence, it was necessary to perform structural examination and molecular docking calculations using both the available forms of IL-22 structures for possible heparin binding.

Based on X-ray diffraction studies, IL-22 has been shown to share strong secondary structural homology with IL-10 and IFN-γ, both heparin-binding family members. The high-resolution, crystallised structure of IL-22 monomer (Figure 3.8) superimposes well on a single domain of IL-10 and IFN-γ dimers respectively (Nagem *Et. al.*, 2002;

T. Xu *Et. al.*, 2005). This monomeric structure of IL-22 contains 6 helices (A-F) arranged in an anti-parallel conformation (Figure 3.9) to form a compact, α-helical bundle protein, as seen in Figure 3.10 (Nagem *Et. al.*, 2002). This contrasts with V-shaped structures of monomeric and dimeric forms of IL-10 and IFN-γ (Zdanov, 2010). Moreover, IL-10 and IFN-γ are intertwined, physiological homodimers shown to bind to receptors with the stoichiometry of 1:2:2 for signalling (Walter & Nagabhushan, 1995; Logsdon *Et. al.*, 2004; Zdanov, 2010). As against, IL-22 dimer is an interfacial homodimer, formed at higher concentrations and is readily dissociable. It is formed by electrostatic interaction of charged surface residues on monomer, IL-22A and monomer, IL-22B (listed in Table 3.1) which results in a compact IL-22 dimer (Figure 3.12) as revealed by crystallographic studies (Nagem *Et. al.*, 2002; Nagem *Et. al.*, 2006). Almost 30% of the total surface area of each monomer is involved in its dimer formation. The resultant interface area in the IL-22 dimer that remains inaccessible is twice as much of any other dimers, generated by the specified crystallographic method (Nagem *Et. al.*, 2002). With regards to receptor binding, IL-22 shares one of the receptors with IL-10. Thus, it recruits one IL-22R1 and one IL-10R2 to form a functional IL-22-receptor complex (Logsdon *Et. al.*, 2004; Bleicher *Et. al.*, 2008; Jones *Et. al.*, 2008a). Comparing the receptor-binding residues in IL-22 (Table 3.2) with the interfacial surface residues (see Table 3.1), this shows that dimerisation results in occlusion of receptor-binding residues. This suggested that monomers of IL-22 were physiologically important (Nagem *Et. al.*, 2002). Moreover, IL-22 monomers were also shown as the functionally active forms using SPR and gel filtration chromatography methods (Logsdon *Et. al.*, 2002).

Table 3.1: Residues at the interface of the IL-22 dimer (Nagem *Et. al.*, 2002; Nagem *Et. al.*, 2006)

Monomer A	Arg 142, Phe 24, Arg 40, Lys 11, Asn 143, Gln 15, Lys 28, Gln 16
Monomer B	Glu 133, Asn 143, Val 50, Ser 31, Asp 135, Ile 42, Lys 28, Ile 146,

Table 3.2: Receptor-binding amino acid residues on monomeric IL-22

IL-22 receptor subunits	Residues involved	Method	References
IL-22R1	Phe 14, Gln 15, Thr 20, Phe 24, Ser 31-Gly 43, Ile 128-Arg 142	Crystal structure of complex IL-22/IL-22R1	(Jones *Et. al.*, 2008a; Bleicher *Et. al.*, 2008)
1L-22R2 (also called IL-10R2)	Tyr 18, Asn 21, Arg 22 on helix A and Tyr 81, Glu 84 on helix D	SPR analysis, Mutagenesis Experiment, Structural homology with ternary complexes of IL-10/IL-10R1/IL-10R2	(Logsdon *Et. al.*, 2004; Jones *Et. al.*, 2008a; Bleicher *Et. al.*, 2008)
1L-22R2 (also called IL-10R2)	Lys 28, Ala 33, Val 50, Arg 55, Pro 80, Phe ala 88, Leu 89, Leu 92	Structural homology to IL-10-IL-10R complexes	(Wu *Et. al.*, 2008)

Given that the nature of GAG-protein interaction is prominently electrostatic and positively charged clusters on protein surface are known GAG-binding sites (D. Xu & Esko, 2014), in the current study, crystallographic structures of monomeric (Figure 3.8 and 3.10) and dimeric forms of IL-22 (Figure 3.12B) were inspected for the surface basic clusters a potential HBSs. Structural inspection of both forms of IL-22 protein revealed the presence of comparatively frequent clusters of acidic residues around basic clusters of interest on the protein surface (Figure 3.8, 3.10 and 3.12B). For instance, a group of basic residues over a short linear sequence of amino acids (^{114}KLKDTVKK^{121}) as observed from the primary sequence of IL-22 (coloured green in Figure 3.1) was seen interrupted by an acidic residue, Asp 117 (coloured red in Figure 3.8) on a three-dimensional structural fold. Because of like-charge repulsion between the acidic residues on the surface of IL-22 and the sulphate groups on heparin chains, this may interfere or prevent the stability of interaction of IL-22 with heparin. Based on structural inspection of both the forms of IL-22, they show lack of distinct

clusters of basic residues that can hold stable heparin interactions and hence, show low likelihood for heparin binding.

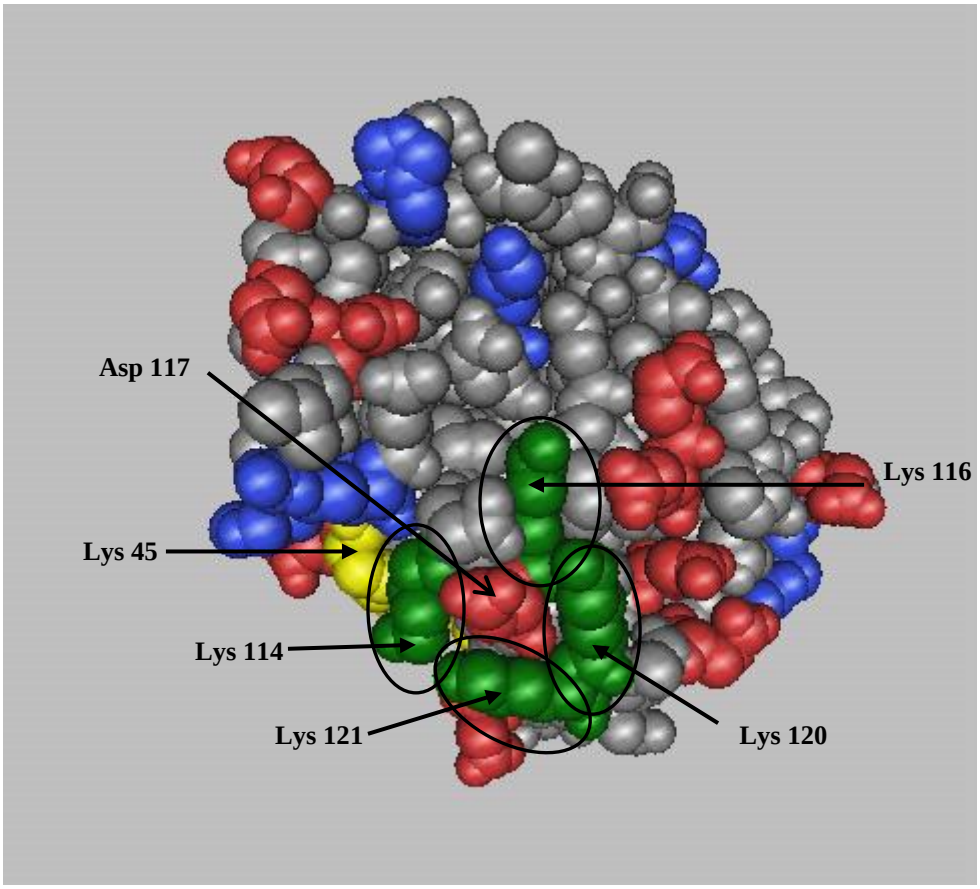


Figure 3.8: Crystal structure of the monomeric human IL-22 (mmdbId: 23387) with a basic cluster interspersed by acidic residues: A crystal structure of monomeric IL-22 is available in the PDB (1M4R). It is viewed and annotated using the Cn3D programme and represented in a space-filled model. The surface residues are colour-coded according to the electrostatic potential: basic residues in blue, acidic in red. Some of the basic residues are coloured in green, circled, and labelled to represent putative heparin binding cluster as highlighted in Figure 3.1 in the primary sequence of IL-22. This cluster is interrupted by acidic residues. Putative basic residues involved in heparin interactions suggested by molecular modelling are coloured yellow. The orientation of this structure is 180^{o} to the left of the central vertical line of axis to the structure view of Figure 3.9.

3.2.2.5 Molecular Docking of Monomeric IL-22 protein with Heparin endecamer ligand

Further, the available crystallographic structure of the IL-22 (monomer PDB:1M4R) (Nagem *Et. al.*, 2002) enabled molecular docking calculations to be performed. A

docking protocol, similar to that previously used on IL-18 was followed. Heparin endecasaccharides (mmdbId: 3448) were systematically docked onto a three-dimensional structure of monomeric IL-22 (mmdbId: 23387) using Autodock 2.4. The favourable orientation of endecameric heparin ligands on the IL-22 structure was observed on the face of helix A and helix D. As shown in Figure 3.9, the key basic residues lie in close proximity and in association with heparin probes were identified as Lys 11, Arg 142, Arg 55 residues at one site and Arg 40 and Lys 45 at the other site. Thus, the two observed heparin binding sites on the surface of helices A/D of the monomeric IL-22 structure seem to act in co-operation as a single heparin-binding site (seen as green sticks in Figure 3.9).

The docking strategy calculated the interaction energy of the best fit complex between the IL-22 monomer and heparin endecameric chains. The predicted intermolecular interaction energy calculated was -1130 kcal/mol. This interaction energy value was slightly lower than a threshold value of -1000 kcal/mol. A value below this threshold indicates an ability of a protein to bind heparin. However, the binding energy for IL-22-heparin complex was relatively higher than the values calculated for experimentally verified, heparin-binding cytokines like IL-12 (-2200 kcal/mol) and IL-10 (-1442 kcal/mol) with pentameric heparin chains (collaborator's personal communication). This could possibly be due to the presence of frequent acidic residues on the surface of the IL-22 monomer. Figure 3.10 is a superimposable view of the Figure 3.9 in a space-filled diagram and reveals the distribution of acidic residues (coloured red) surrounding the basic residues (coloured yellow) that were predicted in heparin binding. Therefore, presence of such acidic residues might also weaken or diffuse the electrostatic potential of surface basic charges resulting in reduced capability of monomeric IL-22 to form stable complexes with heparin. However, further experimental investigation will confirm the heparin-binding property of IL-22.

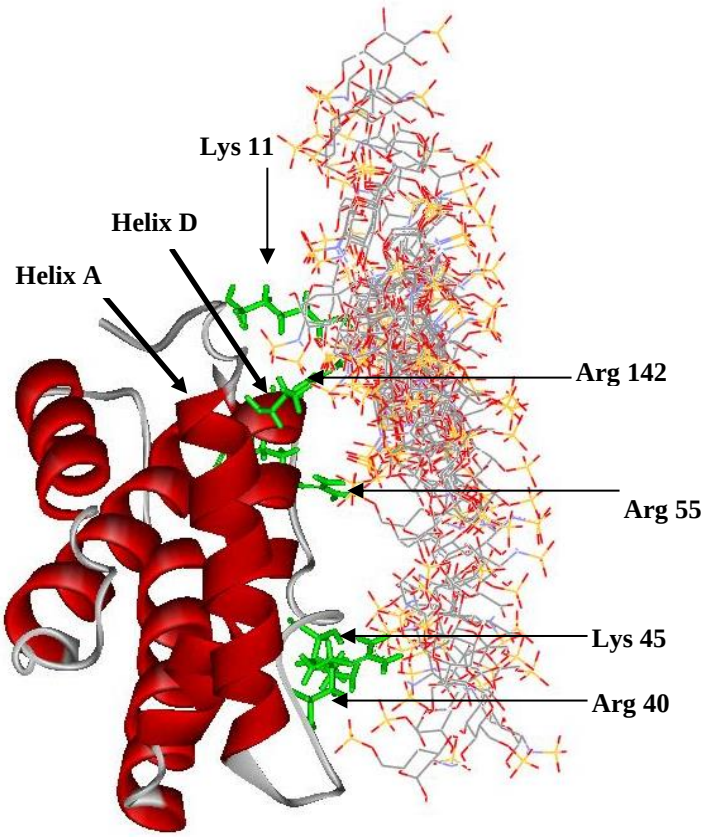


Figure 3.9: Putative Heparin-Binding Sites in monomeric human IL-22 (mmdbId: 23387) interacting with a heparin endecamer: A crystal structure of monomeric IL-22 has been represented as ribbons with helices coloured red and docked with synthetic heparin endecamer as probe with the ten superimposable lowest energy complexes are shown. The basic residues, Lys 11, Arg 142, Arg 55, Lys 45 and Arg 40 were identified as possible parts of the heparin binding site represented as green sticks and the docked heparin chains shown as stick models with carbon-black, oxygen-red, nitrogen-blue, sulphur-yellow.

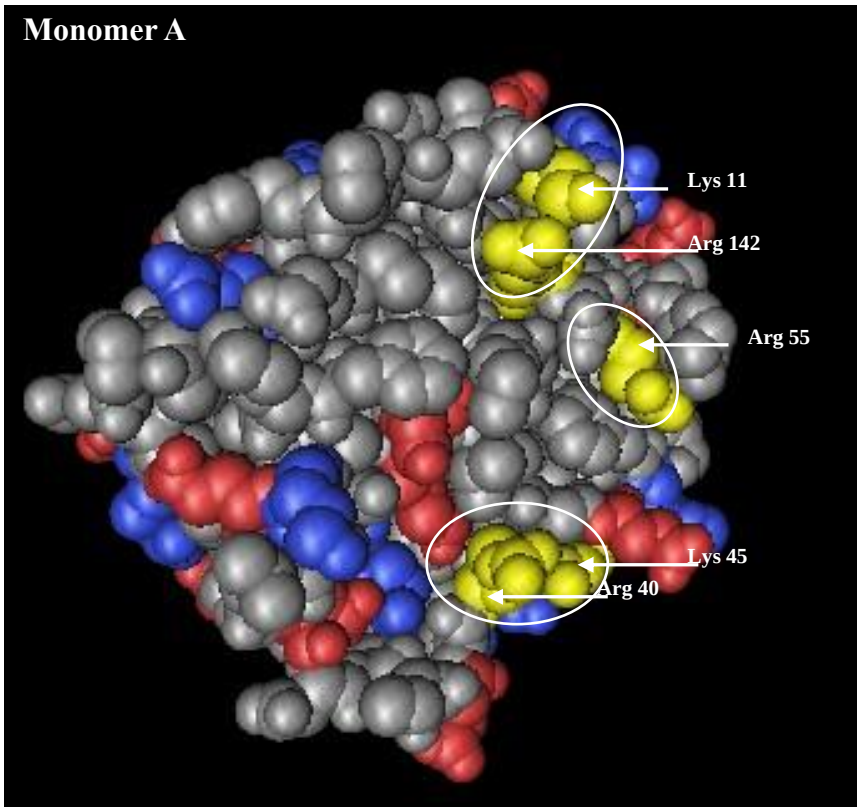


Figure 3.10: Crystal structure of the monomeric human IL-22 (mmdbId: 23387) with putative heparin-binding sites: A crystal structure of monomeric IL-22 is available in the PDB (1M4R). It is viewed and annotated using the Cn3D programme and represented in a space-filled style. The surface residues are colour-coded according to the electrostatic potential, basic residues in blue, acidic in red and putative basic residues involved in heparin interaction either singly or in clusters are coloured yellow, circled and labelled. The orientation of this structure is similar to the upper molecular model structure in ribbon style of Figure 3.9.

3.2.2.6 Molecular Docking of Monomeric IL-22 protein with Heparin pentamer ligand

Generally, a shorter heparin chain identifies a core heparin-binding site, or the minimal size required for heparin chains to interact with the target protein. The IL-22 monomer was also docked with a shorter heparin chain, a pentamer, which further increased the interaction energy of complex to -810 kcal/mol, compared to the interaction energy obtained with endecamaric ligands. This suggested a reduced ability of the IL-22 monomer to bind to pentameric ligands (Figure 3.11). The interaction energy value obtained with the pentameric heparin chains also indicated that the endecameric heparin chains provided an extended binding-site and therefore offers a more favourable interaction with IL-22 compared to shorter pentameric chains. Amino acids contributing to the predicted interaction of IL-22 with the heparin pentamer were Lys 11, Gln 15 on helix A and Asn 143, and Arg 142 on helix D, as shown in Figure 3.11 (Mulloy & Forster, 2008). Whereas, the residues Lys 45 and Arg 40 were additionally recognised by the endecamer, but not the pentamer, as observed in Figure 3.9 and 3.11, respectively. Though the pentameric heparin ligands interacted across the same face of helices A/D as endecameric ligands, the key residues in close contact with the heparin chains were different. In fact, the presence of contact residues Gln and Asn, which are known to play a lesser role in heparin/HS binding than basic residues, Arg and Lys (Hileman *Et. al.*, 1998) were identified in the pentameric interaction and that could have possibly resulted in the increased interaction energy of this complex (IL-22-heparin pentamer).

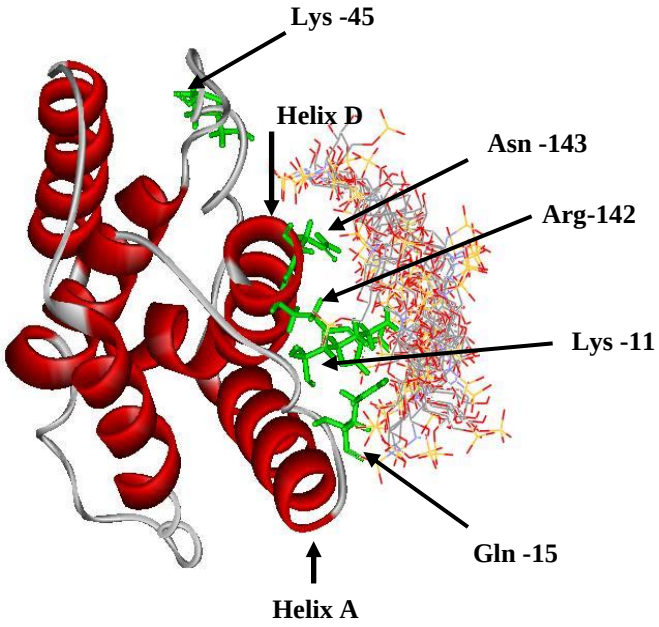


Figure 3.11: Putative Heparin-Binding Sites in monomeric human IL-22 (mmdbId: 23387) interacting with a heparin pentamer: A crystal structure of monomeric IL-22 has been represented as ribbons with helices coloured red and docked with a synthetic heparin pentasaccharide as a probe with the ten super-imposable lowest energy complexes shown. The basic residues Asn 143, Arg 142, Lys 11 and Gln 15 are represented as green sticks and were identified as possible units of interaction with the docked heparin chains shown as stick models with carbon-black, oxygen-red, nitrogen-blue, sulphur- yellow.

Since the monomeric form of IL-22 is biologically active, the prediction of heparin-binding ability of the IL-22 monomer by molecular docking was important. An interesting question that arises from this is whether the putative heparin and the receptor-binding sites on IL-22 monomer coincides? This evaluates whether heparin could possibly interfere with IL-22 signalling and activity through receptor binding. Table 3.2 details the residues on the IL-22 monomer involved in binding to the respective heterodimeric receptor subunits (Logsdon *Et. al.*, 2004; Jones *Et. al.*, 2008a; Bleicher *Et. al.*, 2008). Comparing these residues involved in receptor-binding (from Table 3.2) with those at the putative HBS (Figures 3.9 and 3.11), showed an overlap of few residues at the site of monomeric IL-22. This suggested that heparin might affect the biological activity of IL-22 by competing with its cognate receptors, IL-22R1, for the interaction sites at Gln 15, Arg 40, and Arg 142; and with IL-10R2 at Arg 55.

Additionally, the basic residues predicted in heparin-binding on the IL-22 monomer (Figures 3.9 and 3.11) were compared with the key residues involved in IL-22 dimer formation (see Table 3.1; Nagem *Et. al.*, 2002). Comparison shows that most of the putative heparin-binding residues on the IL-22 monomer were significant components in IL-22 dimerisation. These common residues included Lys 11, Arg 40, Arg 142 and Asn 143, which appears to be concealed at the interface, on dimer formation. Overall, if IL-22-heparin binding is to occur then heparin not only impedes dimerisation, but also competes with IL-22-specific membrane receptors. Such competition for receptor-binding has also been demonstrated for IL-10 (Salek-Ardakani *Et. al.*, 2000; Kunze *Et. al.*, 2016) and IFN-γ (Fernandez-Botran *Et. al.*, 1999; Saesen *Et. al.*, 2013), the heparin-binding members within IL-22 family.

3.2.2.7 Molecular Docking of Dimeric IL-22 protein with Heparin endecamer ligand

Further molecular docking between the dimeric crystal structure of IL-22 and the heparin endecamer using Autodock 2.4 was performed, as shown in Figure 3.12A. Interestingly, molecular docking calculations identified a different binding mode and site on the IL-22 dimer than that observed with the monomer alone. Heparin ligands were found positioned on the IL-22 dimer connecting both the monomeric units and occuping a binding site that spanned across both the units. As seen in Figure 3.12A, specifically lysine residues, Lys-91 and 95 of monomer A and Lys-28 of monomer B were found involved in the heparin-binding interface made by docking. Comparison of the two molecular models, one in which heparin was docked onto the IL-22 monomer (Figure 3.9) and the other onto the IL-22 dimer (Figure 3.12A) revealed that the basic residues predicted for heparin binding were different in both the forms of IL-22.

Docking studies calculated the energy values of interaction between dimeric IL-22 and endecameric heparin ligand, as -1042 kcal/mol. With pentameric ligands, it was calculated as -650 kcal/mol. Overall, the interaction energies for both the forms of IL-22 (monomer and dimer) with heparin endecamers were slightly lower than -1000 kcal/mol. A value below this threshold indicates heparin/HS binding (Mulloy & Forster, 2008). However, the energetics of IL-22-heparin complex were much higher than the known values of experimentally detected, heparin-binding proteins, IL-10 (-1442 kcal/mol). Thus, this predictive data from molecular docking indicated a weak

interaction for both the forms of IL-22 with heparin and hence less likelihood of binding to heparin. Experimental investigation would be required to further confirm the possibility of heparin-binding.

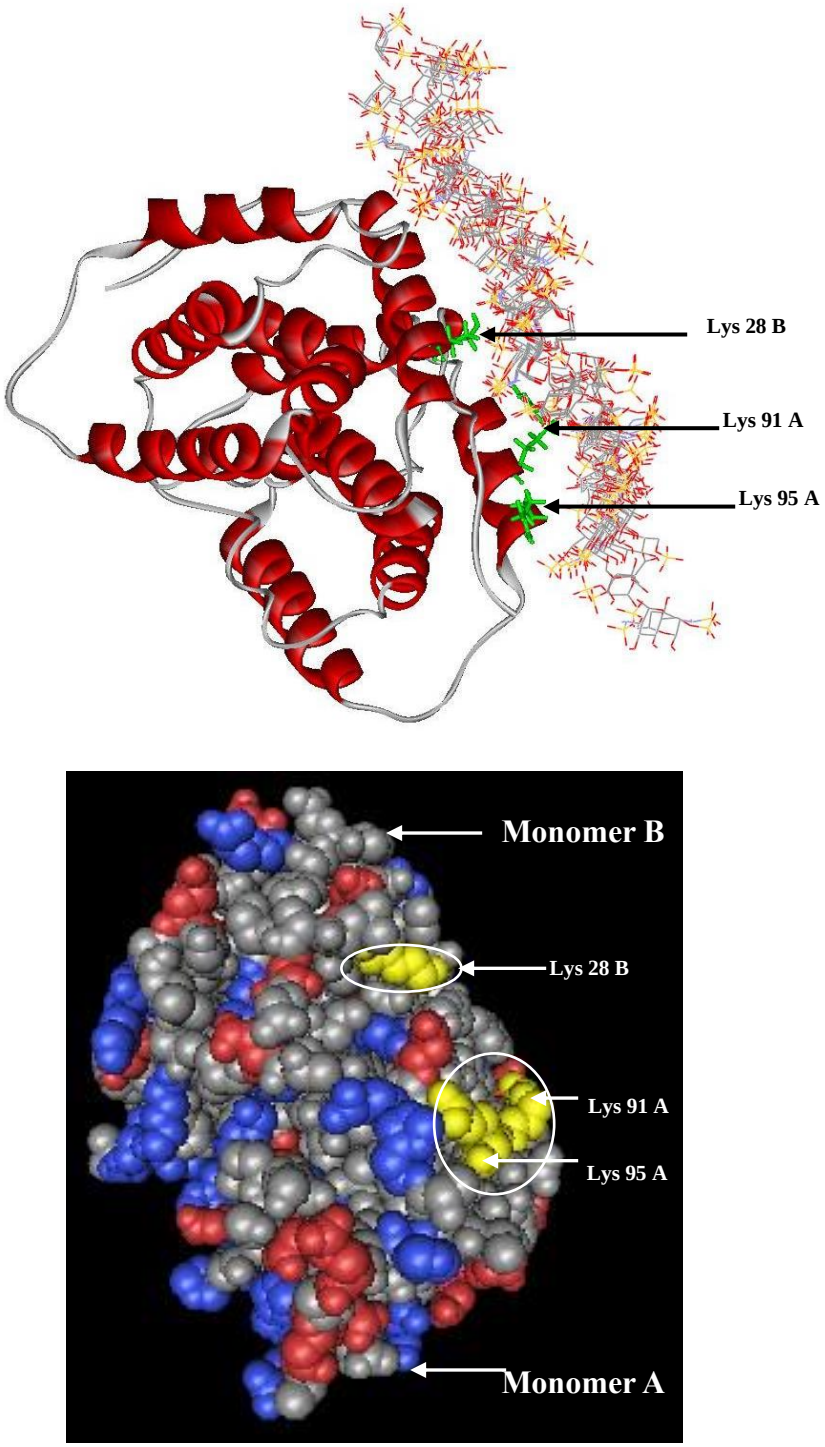


Figure 3.12: Putative Heparin Binding Sites in dimeric human IL-22 interacting with a heparin endecamer: A) A crystal structure of dimeric IL-22 has been represented as ribbons with helices coloured red and docked with a synthetic heparin endecamer as a probe with the ten superimposable lowest energy complexes shown. The lysine residues, Lys 91 and 95 of monomer A with Lys 28 of monomer B were identified as possible parts of the heparin binding site represented as green sticks and the docked heparin chains shown as stick models with carbon-black, oxygen-red, nitrogen-blue, sulphur-yellow. **B)** A crystal structure of dimeric human IL-22 (mmdb Id 23387) is available in the PDB (Id 1M4R). It is viewed and annotated in the Cn3D programme and represented in a space-filled style. The surface residues are colour coded according to the electrostatic potential, basic residues in blue, acidic in red, and putative basic residues involved in heparin interaction as yellow, circled, and labelled. The orientation of this structure is similar to the Figure 3.12A in ribbon style.

3.2.3 Binding of IL-11, IL-18, and IL-22 to Immobilised Heparin-BSA conjugate: ELISA Approach

As pointed out earlier, the molecular docking data balances well with the immobilised heparin-binding ELISA method which provides information on affinity and specificity of heparin-cytokine interactions. Therefore, having performed predictive docking calculations, it was next sought to experimentally examine heparin binding in these proteins. Below is a short summary on this approach and its advantages over other biochemical methods.

This highly sensitive ELISA method was developed in our laboratory and was routinely employed to quantitatively characterise various GAG-cytokine interactions. In principle, this approach is a simple, heparin-capture ELISA (see Section 2.1 for method). Because free GAG chains adsorb poorly compared to proteins onto the plastic surfaces of the ELISA plate, heparin was immobilised by conjugation with BSA. An ELISA plate coated with this conjugate is analogous to an *in vitro* immobilised matrix of heparin that mimicks *in vivo* HS chains presented on proteoglycans. Cytokines that bind to this immobilised heparin are then detected with cytokine specific antibodies. To test for non-specific binding of proteins on to the plastic surfaces of ELISA plates, a mock-conjugated BSA as a control is employed. This is BSA treated with the conjugation reagents but in the absence of heparin, hence, it acts as an internal negative control for heparin-binding experiments (Najjam *Et. al.*, 1997a). This method was validated at the time of its conception using prototypical heparin-binding proteins such as FGF and antithrombin (Najjam *Et. al.*, 1997a). Further, with the success of this approach, additional heparin-binding cytokines, such as IL-2 (Najjam *Et. al.*, 1997a), IL-6 (Mummery & Rider, 2000), IL-12 (Hasan *Et. al.*, 1999), GDNF (Rickard *Et. al.*, 2003), Artemin (ART), Neurturin (NTN) (Alfano *Et. al.*, 2007), β-cellulin (Mummery *Et. al.*, 2007), and BMP-7 (McClarence, 2011) were determined.

The immobilised heparin-binding ELISA was mainly used to determine binding at physiological pH and ionic strength. Because, under low ionic conditions, any positively-charged basic cluster on the surface of a protein could attract and bind to heparin. Therefore, the binding that is retained even at physiological conditions is most likely to have a biological function. The sensitivity of this ELISA method was found

equally comparable to affinity co-electrophoresis (ACE) and affinity chromatography (Najjam *Et. al.*, 1997a). More to the advantage of this method has been the use of non-radioactive reagents that have longer shelf-life. Thus, this heparin-binding ELISA has wide applicability with minimum requirements in the study of heparin-cytokine interactions.

3.2.3.1 Validation of Newly Synthesised Heparin-BSA Conjugate with Immobilised Heparin Conjugate using GDNF

The efficacy of this newly synthesised heparin-BSA conjugate and its control mock-conjugated-BSA in heparin-binding studies was initially tested through binding of GDNF. GDNF is a well-studied cytokine with a high affinity for heparin (Rickard *Et. al.*, 2003). Increasing doses of GDNF (0-20ng/ml) were added to ELISA plate wells coated overnight with either heparin-BSA conjugate or the mock-conjugated BSA. As seen in Figure 3.15, GDNF binds to heparin-BSA in a dose-dependent manner and binding was detectable with as little as 5ng of GDNF. Even though some binding of GDNF was observed in the absence of heparin, in the wells coated with mock-conjugated BSA, this background binding was considerably lower and showed low dose-dependency. Since these results with GDNF agreed with those obtained using a previous batch of heparin-BSA conjugate, the current newly synthesised batch of heparin-BSA complex demonstrated the required binding characteristics.

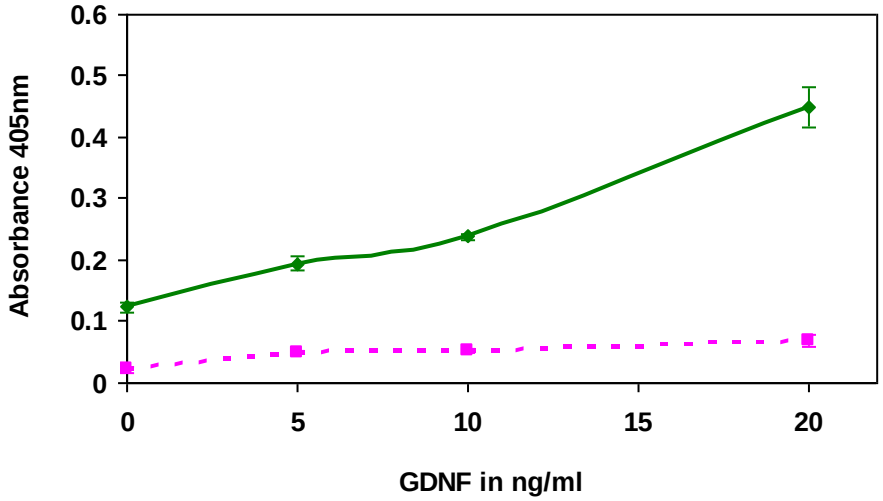


Figure 3.13: ELISA of GDNF with immobilised heparin-BSA conjugate: Increasing concentrations of GDNF were incubated in wells coated with either heparin-BSA conjugate (solid line with ♦**)** or mock-conjugated BSA (dotted line with ■) and the ELISA plate was then read at 405nm. GDNF was used as a positive control for heparin binding using newly synthesized heparin-BSA conjugate. Results represent the mean of triplicate wells at each point, with error bars showing ±SEM. One representative experiment of three independent experiments performed is shown here.

3.2.3.2 ELISA of Interleukins 11, 18 and 22 with Immobilised Heparin Conjugate

Having validated the heparin-BSA conjugate through the study of GDNF, the binding of interleukins 11, 18 and 22 to heparin was then determined. However, prior to performing this experimental heparin-binding assay, a simple ELISA was performed for each interleukin under study to detect the activity of antibody used for the assay. This ELISA did not involve use of heparin-BSA or mock-BSA conjugate. The interleukins were bound directly to adsorbent plastic surfaces of the wells and, after blocking, the respective antibodies were used to detect this binding. Increasing concentrations of each interleukin were added to the ELISA wells followed by incubation with their corresponding antibodies. Colour development was observed on addition of substrate, with dose dependent increase in absorbance values at 405 nm, which is shown as a solid straight line in Figures 3.14, 3.15, 3.16 for IL-11, IL-18 and IL-22, respectively. This suggested that the antibodies employed were reliable for the detection of different concentrations of individual interleukins under the conditions of study.

The heparin-binding ELISA technique was now used to study binding of IL-11 to heparin. 25ng/well concentrations of heparin-BSA and mock-BSA conjugate were coated onto an ELISA plate, and increasing concentrations of IL-11 (0-20ng/ml) were added to the respective wells. Figure 3.14 shows that, despite strong reactivity of anti-IL-11 with IL-11, there was only a trivial difference in absorbance values between IL-11 concentrations to heparin-BSA conjugate and to mock-BSA conjugate. This minimal difference between IL-11 binding to heparin-BSA conjugate and internal control without heparin clearly showed that IL-11 was not binding to heparin.

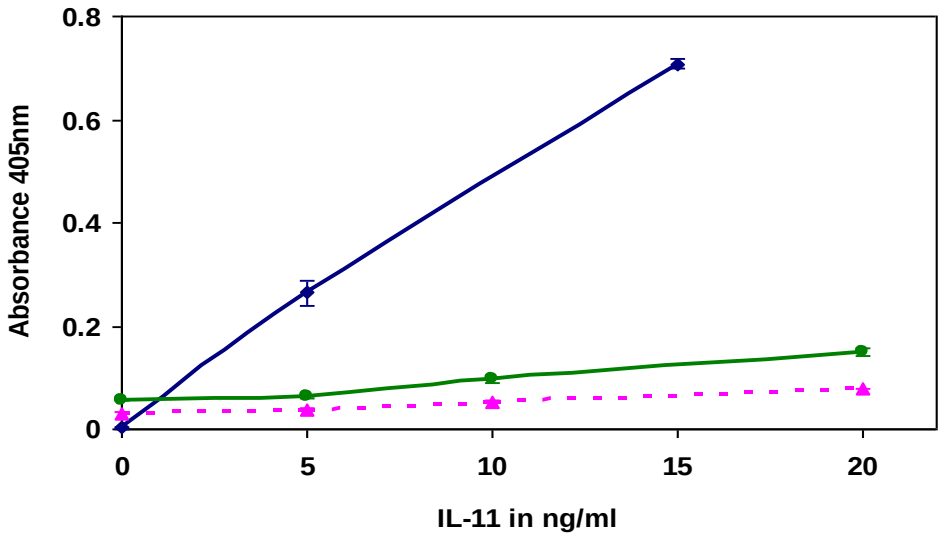


Figure 3.14: ELISA of IL-11 not binding with immobilised-heparin conjugate: IL-11 absorbed directly on ELISA plate wells shows dose dependent increase in absorbance values at 405nm indicating binding of IL-11 is readily detectable with anti-IL-11 (♦). Binding of IL-11 to the heparin-BSA conjugate (solid line with ●**)** and mock-conjugated BSA (dotted line with ▲) coated surfaces of ELISA wells seen as overlapped absorbance curves. Results represent the mean of triplicate wells at each point, with error bars showing ±SEM. One representative experiment of three independent experiments performed is shown here.

The optimised ELISA technique for heparin-binding was also employed for IL-18. Irrespective of the IL-18 concentrations (5, 10, and 20ng/ml) used to incubate with heparin-BSA and mock-BSA conjugate, almost super-imposable absorbance values were obtained (Figure 3.15). This result also concluded that IL-18 had no affinity for heparin.

Lastly, the ELISA results with IL-22 also showed no difference in observed absorbance values for increasing doses of IL-22 with heparin-BSA conjugate binding and background binding in the absence of heparin, i.e., mock-BSA conjugate (Figure 3.16). This indicated that IL-22 does not bind to heparin. IL-22 has been known to dimerise at higher concentrations and dimers have been detected at concentrations around or > 1 µg/ml (de Oliveira Neto *Et. al.*, 2008). In the current experimental study, low concentrations of IL-22 at 50-200ng/ml and pH 7.4 were used which suggested that IL-22 tested was predominantly in monomeric form. This biologically active monomeric form of IL-22 had no affinity for heparin.

Thus, in-line with the predictive molecular docking results, the ELISA assay provided experimental evidence for no detectable binding of IL-11, IL-18, and IL-22 to heparin at physiological pH and ionic strength using this approach.

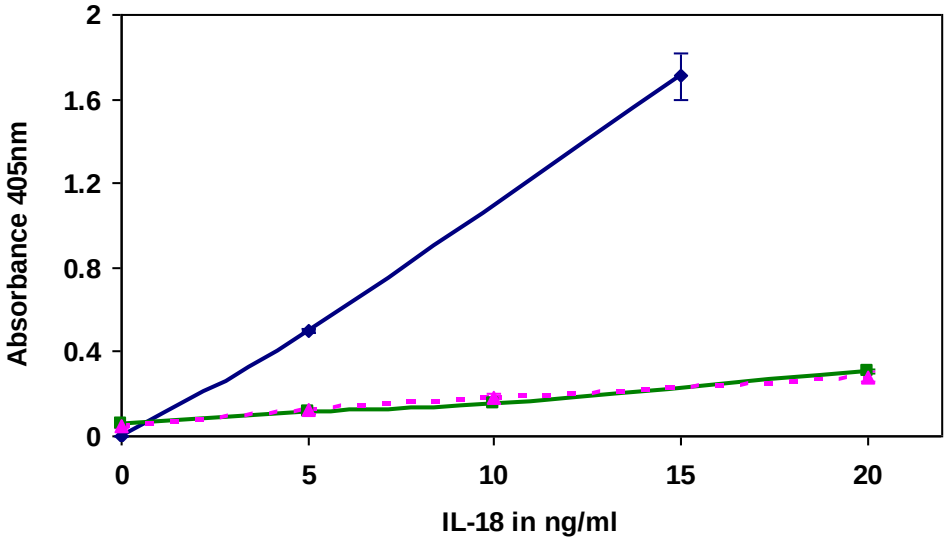


Figure 3.15: ELISA of IL-18 not binding with immobilised heparin-BSA conjugate: Direct binding of IL-18 with anti-IL-18 on surfaces of ELISA plate wells is shown as a dose dependent increase in absorbance values at 405nm representing solid line (♦). Binding of IL-18 to the heparin-BSA conjugate (solid line with ■**)** and mock -conjugated BSA (dotted line with ▲) coated ELISA wells seen as almost indistinguishable absorbance curves. Results represent the mean of triplicate wells at each point, with error bars showing ±SEM. One representative experiment of three independent experiments performed is shown here.

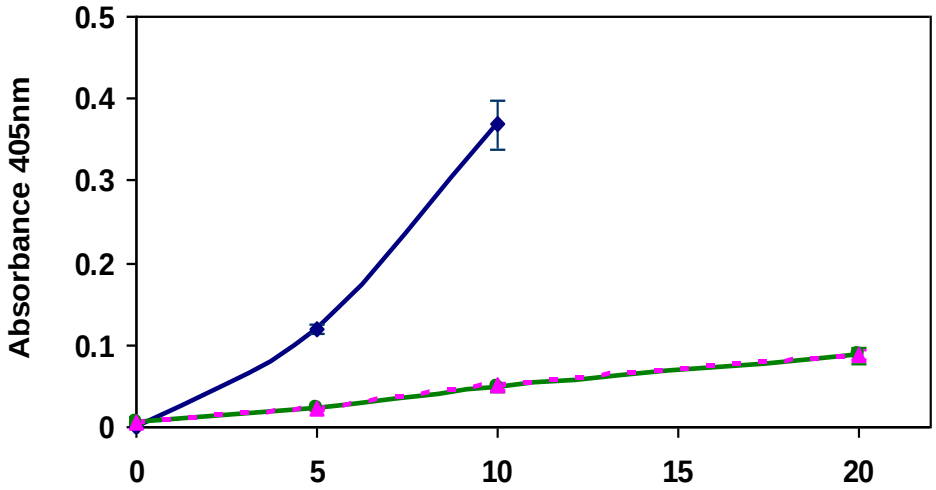


Figure 3.16: ELISA of IL-22 not binding with immobilised heparin-BSA conjugate: IL-22 absorbed directly on surfaces of ELISA plate wells was readily detected by anti-IL-22 as shown by a dose dependent increase in absorbance values at 405nm, solid line (♦). Binding of IL-22 to the heparin-BSA conjugate (solid line with ■**)** and mock -conjugated BSA (dotted line with ▲) coated overnight on ELISA wells seen as overlapped absorbance curves. Results represent the mean of triplicate wells at each point, with error bars showing ±SEM. One representative experiment of three independent experiments performed is shown here.

3.3 Discussion

The aim of the current study was to determine whether the interleukins, IL-11, IL-18 and IL-22 belonging to different classes of cytokines bind to heparin/HS. They were selected based on structure-function relationships with their family members that exhibited heparin-binding activity. A strategy of a combination of various predictive approaches such as analysis of primary sequences, three-dimensional structures and molecular docking methods were employed that predicted heparin-binding sites on the surface of the selected interleukins. Further and experimental assay, the heparin-binding ELISA was successfully used to validate and support the predicted data. Most surprisingly, the results from this study demonstrated that none of the three selected interleukins bound to heparin while their respective family members were known heparin-binding cytokines.

IL-11 did not exhibit measurable affinity for heparin binding, despite, containing a basic sequence motif (^{111}RLDRLLRR^{118}) that closely resembled a Cardin-Wintraub motif (BBXB) in its primary sequence. As mentioned earlier, most consensus sequence motifs contain basic residues in linear arrangement with hydropathic residues (X) such as Asn (N), Ser (S), Gly (G), Ala (A), Leu (L), Ile (I), Tyr (Y) (Hileman *Et. al.*, 1998; Gandhi & Mancera, 2008). These polar and hydrophobic amino acids in heparin-binding domain have been shown to stabilize the interaction by formation of additional non-ionic bonds. However, if acidic residues, Asp (D), Glu (E), are present instead of the above-mentioned hydropathic residues (X) then these interactions destabilize due to ionic-charge repulsion with polyanionic heparin chains (Hileman *Et. al.*, 1998; Gandhi & Mancera, 2008). In case of IL-18 (^{129}KERDLFKLILKK^{140}) and IL-22 (^{114}KLKDTVKK^{121}), although the clusters of basic residues near the carboxyl-terminus of their primary sequences (Figure 3.1), did not match any consensus sequence motifs, these basic peptide sequences contained strong acidic residues such as glutamic acid (E) and asparatic acid (D) as adjacent residues. When the three-dimensional structures of these interleukins, IL-18 and IL-22, were examined these acidic residues were found to be surface-exposed which could have a disturbing effect on heparin interaction. Thus, one of the explanations to the lack of heparin-binding ability in these interleukins could be the position and influence of acidic residues that reduces the likelihood of their basic clusters to be part of an HBD. Collectively, analysis of the primary

sequences of interleukins IL-11, IL-18 and IL-22 enriched in basic residues support the documented views that the heparin/HS-binding sites in proteins are not always or necessarily defined by a unique sequence or a structural motif (Forster & Mulloy, 2006; Mulloy & Rider, 2006; Mulloy & Forster, 2008; D. Xu & Esko, 2014; Gallagher, 2015). More recently, structural studies in a subset of Neutrophil-activating chemokines were shown to have unique GAG binding sites formed by conserved and chemokine-specific amino acids that could not be predicted from primary sequence alone. Rather a 3D structure determined the geometry and topology of the unique GAG-binding surface of each individual chemokine (Rajarathnam *Et. al.,* 2018).

Additionally, these three interleukins studied were of human origin and there is high amino acid sequence homology observed between the human and mouse interleukins, such as 88% in IL-11 (J. C. Morris *Et. al.*, 1996), 64% in IL-18 (Ushio *Et. al.*, 1996), and 80.8% in IL-22 (Wolk & Sabat, 2006a). Therefore, it can be expected that the absence of heparin-binding characteristic would be retained across another mammalian species. While, all the selected set of interleukins failed to exhibit binding with heparin/HS on experimental testing, it is interesting to understand the reasons underlying this observation by comparing the structural details of putative heparin-binding domains in them with the characterised or well-studied HBDs of members of their respective family that are known to interact with heparin/HS.

IL-11 along with IL-6, CNTF, LIF, OSM, CT-1 belongs to a group of structurally and functionally related cytokines termed as IL-6 type or gp-130 receptor cytokines (Kishimoto *Et. al.*, 1994; Heinrich *Et. al.*, 2003; Negahdaripour *Et. al.*, 2016). All members of this IL-6-type family cytokines signal through activation of a common receptor-subunit, gp-130 (Dahmen *Et. al.*, 1998). With regards to heparin/HS binding within this family, LIF has not yet been experimentally determined to bind heparin. However, docking calculations of LIF has shown strong predictions to be able to interact with heparin. The plausible heparin-binding site on LIF was shown to be located at the end of helix C (Arg 133, lys 137) and along the length of helix A (N 25, Q 26, R 28) that overlaps one of the receptors, gp-130 binding sites (Mulloy & Forster, 2008). In contrast, another member of the gp-130 receptor family, CNTF (ciliary neurotrophic factor), was predicted to lack heparin-binding ability, based on docking calculations. Also, CNTF has not been experimentally reported to bind heparin so far (Mulloy & Forster, 2008). However, based on current findings from biochemical

experiments, it is concluded that IL-11 does not bind to heparin/HS. Recently, the first crystallographic structure of IL-11 was published (T. L. Putoczki *Et. al.*, 2014) which allowed us to examine the structure and to identify surface-exposed basic residues (mainly Arg) in the predicted heparin-binding region in IL-11. Before the availability of this IL-11 structure, the homology models of IL-11 were used as a template for structural study (Section 3.2.2.1, Figure 3.2) (M. J. Czupryn *Et. al.*, 1995a; Tacken *Et. al.*, 1999). These structural models were proposed based on sequence alignment or common secondary structural features with other members of family. As expected, the predicted heparin-binding region derived from the homology models of IL-11 corroborated with the experimentally determined structure of IL-11. Nevertheless, it still remains unanswered why IL-11 does not bind to heparin/HS, despite of this interleukin possessing an array of basic amino acids (mainly arginine) in primary sequence. This cluster of Arg residues was also observed as a surface element, on the three-dimensional crystal structure of IL-11 (Figure 3.2) (T. L. Putoczki *Et. al.*, 2014). One limitation to this understanding is the lack of molecular modelling data which would have enabled to gain an insight into whether these basic residues could form a region of high positive charge density and energetics to be able to engage GAG chains.

Since the structure of IL-11 is solved (T. L. Putoczki *Et. al.*, 2014), it is useful in highlighting the difference in heparin-binding ability of IL-11 and its close structural and functional homologue, IL-6. IL-6 is a well-characterised, heparin-binding counterpart of IL-11 within this family. In case of IL-6, strong predictions to bind heparin by molecular docking calculations was confirmed by experimental studies (Mummery & Rider, 2000). Moreover, this predictive method located the heparin-binding domain on IL-6, as a composite site with basic residues from both the helices A (K 27, R 30, K 66) and D (K 171, Q 175, R 179, R 182) forming a shallow groove (Mulloy & Forster, 2008). Essentially, this binding site was devoid of acidic residues in the neighbouring units that could generate any like-charge repulsion and/or steric hindrance (Mulloy & Forster, 2008). It also fulfilled the typical requirement of 4-6 basic residues, Arg (R) or Lys (K) that formed a surface basic cluster on the three-dimensional structure of IL-6 (Hileman *Et. al.*, 1998). Since GAG-protein interactions are predominantly electrostatic in nature, it was important to understand the spatial distribution of charges. A recent report analysed the distribution of surface electrostatic potential on IL-6 (1alu.pdb) (Somers *Et. al.*, 1997) and IL-11 (4mhl.pdb) structures (T. L. Putoczki *Et. al.*, 2014). This study showed that IL-6 displayed more localised

patches of positive electrostatic potential on its protein surface (T. L. Putoczki *Et. al.*, 2014), which indeed are in agreement with the regions of heparin binding in IL-6 (Mummery & Rider, 2000; Mulloy & Forster, 2008). However, in comparison, IL-11 showed larger but diffused spread of positive electrostatic potential (T. L. Putoczki *Et. al.*, 2014). Apparently, it seems in IL-11 that basic residues distant in sequence do not come close to form densely packed clusters on higher structural folding, except on helix C that contained cluster of surface Arg residues representing Cardin-Weintraub motif (^{111}RLDRLLRR^{118}). Of note, it is recently shown by a study that all surface basic residues, do not possess equal and high electrostatic potential to be identified as GAG-binding site in proteins whether they follow Cardin-Weintraub rule or not (Sarkar & Desai, 2015). Thus, compared to IL-6, IL-11 appears to exhibit a weak electrostatic potential near its surface of basic charges in the proposed heparin-binding region, possibly explaining its inability to engage heparin chains.

As mentioned, besides the structural resemblance, IL-11 also shares biological functions with IL-6 (Negahdaripour *Et. al.*, 2016). It closely resembles the hexameric signalling-complex formation in IL-6, including two cytokine specific α-chain receptors and two gp-130 molecules (Barton *Et. al.*, 2000). The experimental evidence from soluble receptor-binding and heparin-binding studies with IL-6 suggested that the proposed HBS was close to but did not overlap with IL-6 receptor-binding sites, IL-6Rα and gp-130. So, heparin interaction may sterically restrict binding of IL-6 to both of its receptors (Mulloy & Forster, 2008). Supporting this, heparin was shown to weakly inhibit IL-6-srIL-6Rα complex formation but strongly inhibited the interaction of this complex with soluble gp-130 receptors (Mummery & Rider, 2000). Hence, the heparin-binding property in IL-6 functionally impacts its signal-transduction events. Given that IL-11 and IL-6 share number of important biological functions along with dominant role of IL-11 over IL-6 in GI cancers (Putoczski *Et. al.*, 2013), the difference in the heparin-binding property between these cytokines could influence their therapeutic role (as discussed later in Chapter 6). Collectively, it can be concluded from this study that heparin-binding is not a common property in the family of gp-130 receptor cytokines.

IL-18 shares the three-dimensional, β-trefoil structural fold with a known family of heparin-binding cytokines, the FGFs (Kato *Et. al.*, 2003). Interestingly, superimposition of the β-trefoil structural scaffold of all FGFs shows almost

overlapping heparin-binding regions in them (Raman *Et. al.*, 2003; R. Xu *Et. al.*, 2012; Y. Li *Et. al.*, 2016). Within this family, FGF-2 is an extensively studied example of a heparin-binding protein (Ornitz & Itoh, 2001a; Ori, 2009; Platonova *Et. al.*, 2014). Despite sharing similar architecture, one notable difference between the HBDs of IL-18 and FGF-2 is the presence of group of acidic amino acids in IL-18. Unlike in FGF-2, these acidic residues in IL-18 were found located within as well as surrounding the cluster of basic components of the proposed heparin-binding domain (Figure 3.17). Findings from the current study, further highlights the important differences in the formation of the HBD that corresponds to the difference in their heparin-binding capacity.

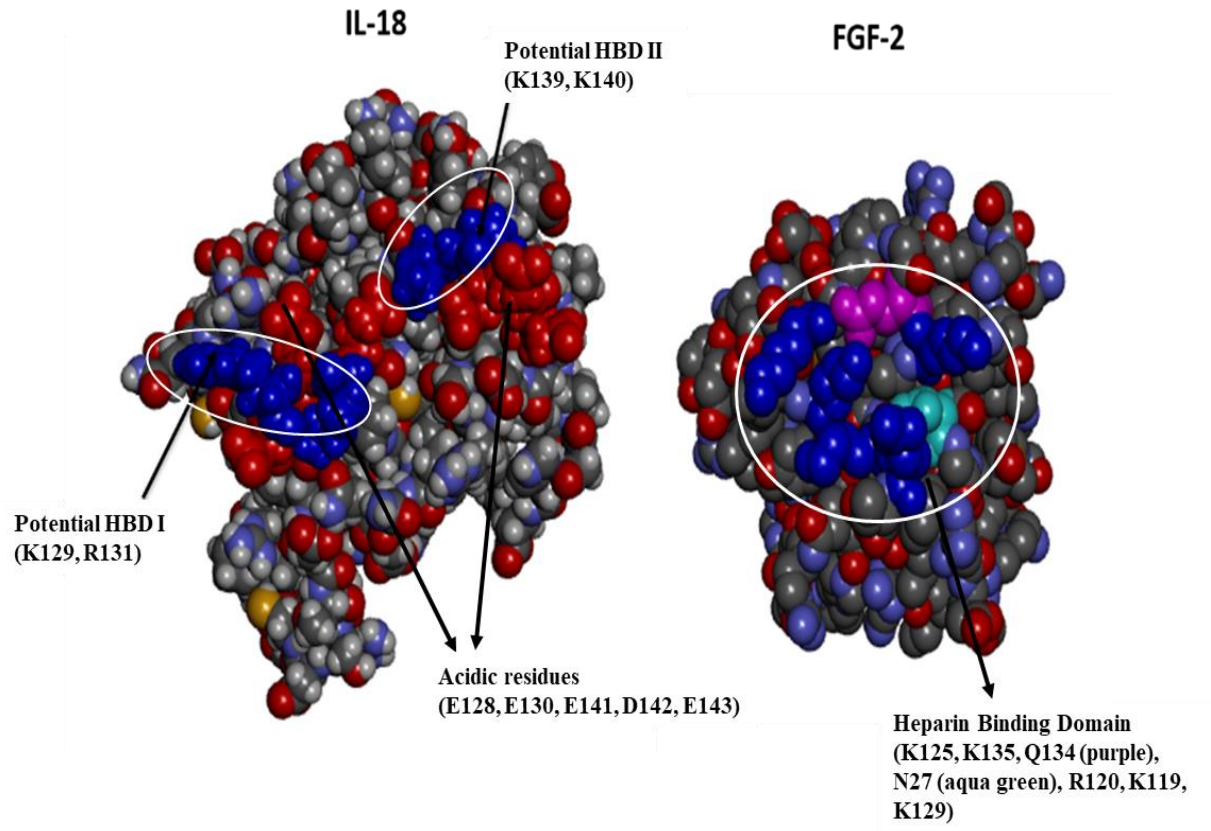


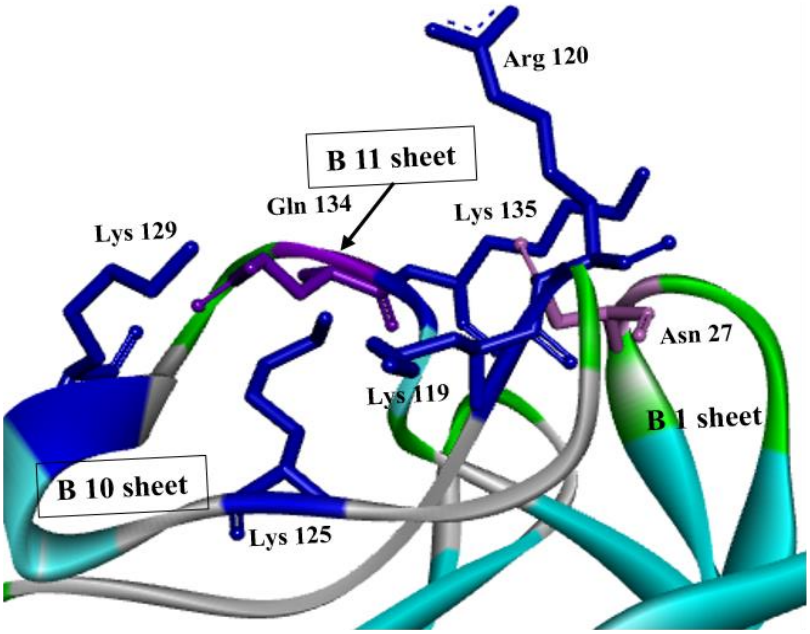
Figure 3.17: Surface view of the β-trefoil structured cytokines, IL-18 and FGF-2 with their heparin/HS-binding domains: A structural overview is presented here to show a notable difference in spatial arrangement of basic residues within the heparin-binding domains of IL-18 and FGF-2. A concentrated cluster of basic residues (blue) devoid of acidic residues (red) is seen in FGF-2 along with support from glutamine-Q134 (purple) and asparagine-N27 (aqua green) residues from surrounding (L. D. Thompson *Et. al.*, 1994; Faham *Et. al.*, 1996). In contrast, IL-18 shows two dispersed basic clusters (HBD I and HBD II) due to interfering acidic residues as neighbouring units. These images in space-filled representation were generated from the PDB structures of IL-18 (1J0S) and FGF-2 (1FQ9) using the Accelyrs Discovery Studio software package.

In the β-trefoil scaffold of FGF-2, the HBS is a discontinuous binding-site formed from the β-strands, β10, β11 and the loop region between them (β10-11 loop) (L. D. Thompson *Et. al.*, 1994; Faham *Et. al.*, 1996; Ori, 2009; R. Xu *Et. al.*, 2012; Nunes *Et. al.*, 2016). Additionally, a stretch of basic residues (β11-12 loop) near the carboxyl-terminus is also involved in the interaction with heparin (Faham *Et. al.*, 1996). The HBD on FGF-2 has been well-studied fromcrystallographic structures of FGF-2 in-complex with heparin hexasaccharides, which identified characteristic conformational properties of the HBS in FGF-2 (Faham *Et. al.*, 1996). As shown in Figure 3.18, it consists of a narrow groove containing a basic residue, Lys 125 at the base. This residue is shown to be critical in the interaction with specific *N*-sulphate and 2-*O*-sulphate groups within the heparin chain. Moreover, the groove is surrounded by other basic

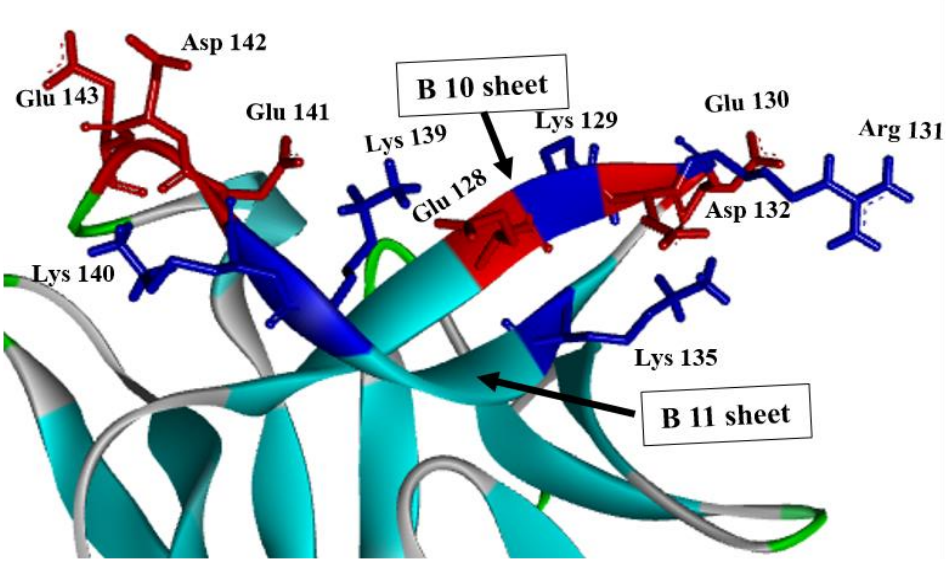
residues and polar amino acids close to the carboxyl-terminal region (Figure 3.18), K 135, Q 134, N 27, R 120, K 119 and K 129 that further favours ionic interactions and van der Waals contacts with sulphate and carboxylate groups of the heparin/HS chains (L. D. Thompson *Et. al.*, 1994; Raman *Et. al.*, 2003; R. Xu *Et. al.*, 2012; Gallagher, 2015). Overall, the topology of the HBS formed by β-strands and the spatial arrangement of basic residues with their side-chains within and around the site in FGF-2 is devoid of any surface acidic residues (as seen in Figure 3.17 and 3.18). Therefore, such a heparin-binding domain sets to maximize surface contact and strong interaction of the protein with heparin chains.

Like FGF-2, IL-18 maintains an overall β-trefoil structural conformation and contains a cluster of basic residues in its primary sequence near the carboxyl-terminus. Indeed, these basic residues originate from similar regions as in FGF-2, comprising of β-strands, β10 and β11 and the connecting loops β10-11 and β11-12. However, on inspection of the three-dimensional structure of IL-18 (Kato *Et. al.*, 2003), this cluster of linearly contiguous basic residues was seen to be disrupted into two smaller pockets/sites of potential heparin-binding, HBD I- K 129, R 131 and HBD II- K139, K140 (Figure 3.17 and 3.18). Moreover, these two sites were interrupted and spatially separated by adjacent acidic residues (E 128, E130, E 141, D 142 and E 143) (as shown in Figure 3.18) that perhaps prevent the formation of a co-operative binding-site for heparin. The presence of such GAG-repelling surfaces separating clusters of basic residues on protein structures suggest a feature not so favourable to GAG binding. This observation was also validated by the molecular docking method. Indeed, computational docking of heparin chains onto the surface of IL-18 predicted an additional binding site formed by the loop regions of β strands, β4-β5 and β7-β8. However, this predicted HBD comprised of only two basic residues, K 53 and K 93 (HBD III) that may not be sufficiently strong to hold a stable interaction with heparin. Several studies on heparin-binding proteins and peptides have indicated clusters comprising of four to six basic amino acids that constitute a HBD on the surface of proteins [as comprehensively reviewed in (Hileman *Et. al.*, 1998)]. Consequently, an insufficient number of basic residues defining a HBD in IL-18 could have also resulted in an inability of this protein to bind to heparin chains. Hence, this explains the current findings from biochemical experiments, which demonstrated that IL-18 does not possess any measurable heparin-binding affinity.

In addition to the structural basis, a quantitative basis to this remarkable difference in heparin-binding property between IL-18 and FGF-2 was observed by comparing their interaction energies computed by molecular docking. The intermolecular interaction energy for IL-18 (-200 kcal/mol) was over 15 times higher than FGF-2 (-3500 kcal/mol) suggesting an unstable binding site in IL-18 for interaction with heparin. This clearly illustrates that, even though IL-18 belongs to the same structural family as FGF-2, it does not share the heparin-binding characteristic based on the difference in stability of HBD. Indeed, IL-18 lacks proper clustering of basic residues to form a coherent and accessible heparin-binding domain (as seen in Figure 3.17 and 3.18), which is the key structural feature on a protein surface for affinity towards heparin/HS.



FGF-2



IL-18

Figure 3.18: Comparative view of the molecular surface of a heparin-binding domain (HBD) on structures of FGF-2 and IL-18: Please refer to next page for Figure legend.

Figure 3.18: Comparative view of the molecular surface of a heparin-binding domain (HBD) on structures of FGF-2 and IL-18: A close-up view of the surface cavity formed by the topological arrangement of β-sheets (β10-β-11) is shown for FGF-2 and IL-18. Note that, in FGF-2, key basic (blue) residues, polar residues, Asn/Gln (purple) are involved in formation of the HBD (L. D. Thompson *Et. al.*, 1994; Faham *Et. al.*, 1996). In contrast, the close-up view of the proposed HBD on IL18, formed by the similar regions as in FGF-2, shows the presence of acidic residues (red) around the clusters of basic residues. This difference in molecular surface of the HBDs could reflect in the heparin-binding ability of these proteins, FGF-2 and IL-18. Amino acids with their side-chains are shown in stick form. The β strands are labelled according to the conventional strand nomenclature for FGFs (Faham *Et. al.*, 1998; Ornitz & Itoh, 2001b). These images with ribbon representation of β-sheets were generated from PDB structures of IL-18 (1J0S) and FGF-2 (1FQ9) and annotated using the Accelyrs Discovery Studio software package.

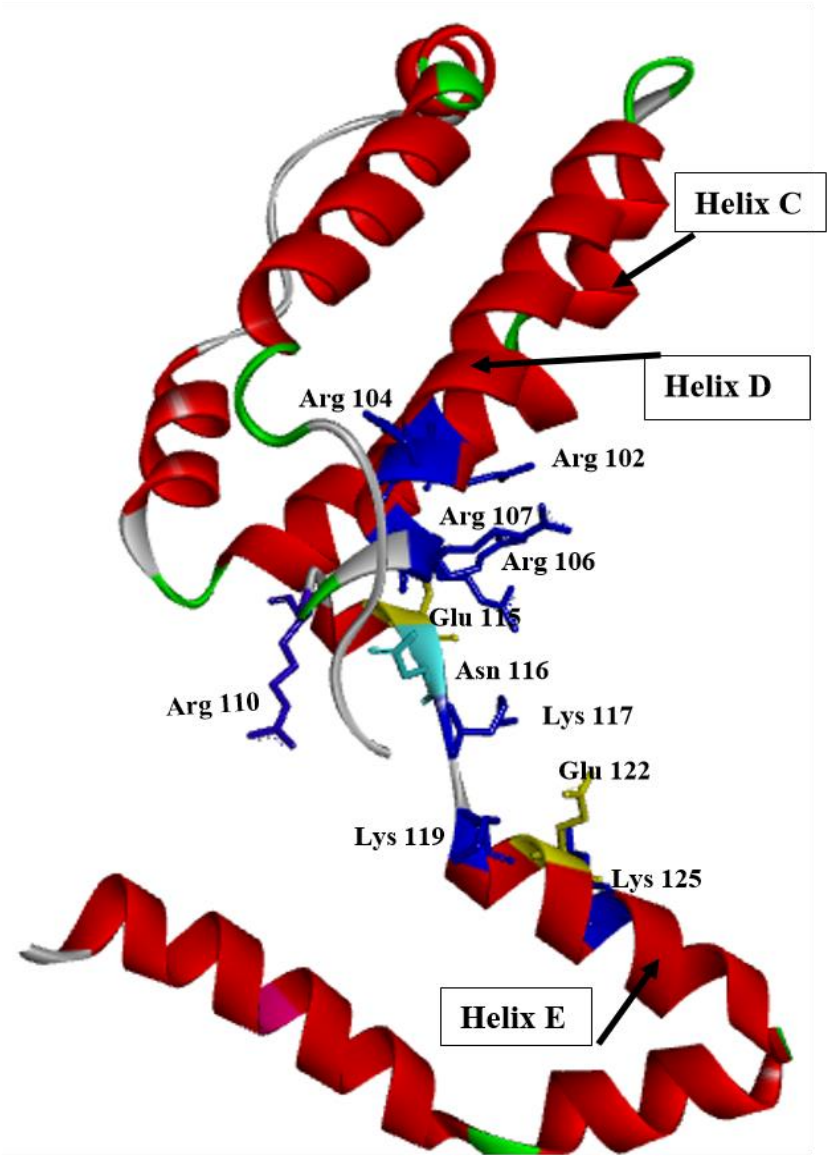
In contrast to the current findings on IL-18, Reeves at al have shown that IL-18 binds to a mixture of GAGs at high concentrations (HS, CS and HA) and at a pH lower than physiological levels (Reeves *Et. al.*, 2010). Since GAG-protein interactions are predominantly electrostatic, low pH conditions can have profound effect on the GAG binding capacity of proteins (Gandhi & Mancera, 2008), as has been seen in case of GM-CSF (Sebollela *Et. al.*, 2005). GM-CSF was observed to undergo conformational changes at low pH compared to neutral pH, which exposes residues like histidines (His) to additionally support surface Lys residues allowing interaction with GAGs (Sebollela *Et. al.*, 2005; Wettreich *Et. al.*, 1999). Reeves *Et. al.* showed that chemical modifications of surface Arg and Lys residues in IL-18 resulted in a significant reduction in binding to GAGs mixture. However, this did not completely eliminate association with GAGs, suggesting a significant role of other basic residues such as His residue (H109) in the observed binding of IL-18 to GAGs at low pH (Reeves *Et. al.*, 2010). This was explained on the basis that as pH falls, His residues become protonated and positively charged to facilitate binding with negatively charged sulphate ions on GAG chains (Gandhi & Mancera, 2008; Reeves *Et. al.*, 2010). Indeed, our findings are more relevant at physiological levels. Another differentiating parameter in the experimental conditions used by Reeves et al is mixture of GAGs. The presence of multiple GAGs in high concentration which mainly occurs at sites of inflammation or pathological conditions, increases the number of different sulphation sites and overall anionic charge density (Bhaskar *Et. al.*, 1998; Solic *Et. al.*, 2005). This in turn may increase the GAG-binding capability of proteins but at the same time may also decrease the level of specificity in GAG-protein interactions. Since heparin/HS is the most generic form of GAGs and represents a physiological ligand to proteins, binding

observed in the presence of different GAGs in combination may be prevalent under certain pathological conditions but cannot be extended to physiological conditions as employed in the current study.

Further, the current study experimentally demonstrated that IL-22 does not bind heparin. This finding was supported by the analysis of the three-dimensional structures of both the IL-22 monomer and dimer, which revealed lack of continuous basic clusters due to a predominance of acidic residues as the neighbouring units. Therefore, this indicated a low likelihood of IL-22 binding to heparin/HS. Additionally, this observation was closely comparable with the molecular docking prediction which suggested a weak interaction of IL-22 with heparin chains. Because IL-22 shares a structure-function relationship with two known heparin-binding cytokines, IL-10 (Salek-Ardakani *Et. al.*, 2000) and IFN-γ (Lortat-Jacob *Et. al.*, 1991b), it is necessary to understand why and how IL-22 differs from these cytokines in the ability to bind heparin.

Despite, similar structural architecture of IL-22 with IL-10 and IFN-γ, it can be seen that the IL-22 monomer (Figure 3.20) is a compact helical structure as opposed to the 'V' shaped structure of monomeric IL-10 (Figure 3.19) and IFN-γ (Nagem *Et. al.*, 2002). In fact, the presence of a specific second disulphide bridge in IL-22 results in flexibility of the loop DE that allows helices E and F to fold back in the groove formed by helices A and D of the same polypeptide chains, resulting in a bundled protein (Figure 3.20) (Nagem *Et. al.*, 2006). Whereas, in IL-10 (Walter & Nagabhushan, 1995; Zdanov *Et. al.*, 1995; Zdanov *Et. al.*, 1996; Zdanov, 2010) (Figure 3.19A) and IFN-γ (Ealick *Et. al.*, 1991), lack of this disulphide bond makes the loop DE more rigid and helices E and F folds as a separate domain resulting in open 'V' shaped monomers (Nagem *Et. al.*, 2002; Nagem *Et. al.*, 2006).

A] IL-10 monomer



B] ^{101} LRLRLRRCHR FLPCENKSKA VEQVKNAFNK ^{130}

Figure 3.19: Ribbon representation of monomeric IL-10 structure to illustrate the location of heparin-binding domain with molecular details: Please refer to next page for Figure legend.

Figure 3.19: Ribbon representation of the monomeric IL-10 structure to illustrate the location of the heparin-binding domain with molecular details (A) Detailed view of the molecular surface of predicted heparin-binding domain on IL-10 monomer (Mulloy & Forster, 2008; Kunze *Et. al.*, 2014; Kunze *Et. al.*, 2016). IL-10 monomer is comprised of six helices A-F. The basic (blue), acidic (yellow) and polar uncharged (aqua green) residues within and around the proposed region of HBD is highlighted. As seen, the basic residues concentrated in the suggested heparin/HS binding region are Arg-102, -104, -106, -107, -110, Lys-117, -119 along with Asn-116 from the C-terminal region of helix D and E on IL-10 monomer. These residues apparently form a surface cluster, encompassing the helices D, E and loop DE. Amino acid sequence of this region near C-terminus is shown below (B) and contains a linear array of basic residues (Arg, R and Lys, K). This sequence is taken from (Salek-Ardakani *Et. al.*, 2000). Amino acids with their side-chains are shown in stick form. These images with ribbon representation of α-helices were generated from PDB structure of IL-10 (2ILK). IL-10 monomer was edited and annotated from its dimer structure (2ILK) (Zdanov *Et. al.*, 1996) using the Accelyrs Discovery Studio software package.

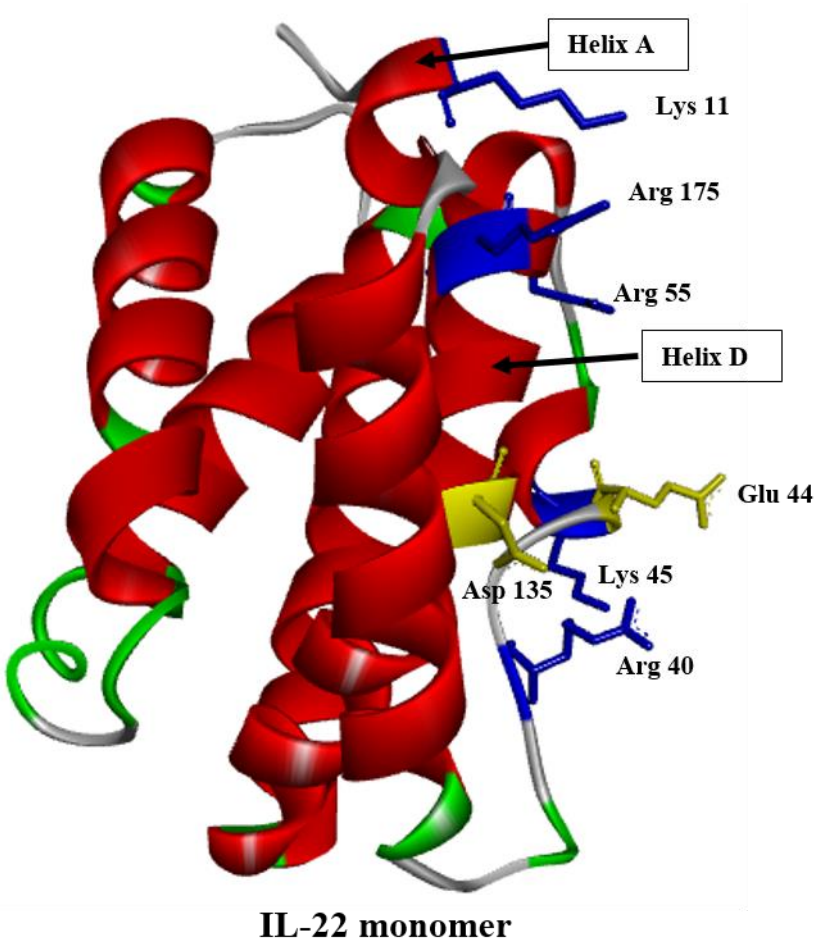


Figure 3.20: Ribbon representation of the monomeric IL-22 structure to illustrate the location of the HBD with molecular details. A detailed view of the predicted HBD on the face of helix A and D of IL-22 monomeric structure by the molecular docking is shown. The basic (blue) residues contributing to the proposed HBD and acidic (yellow) residues within and around this region is shown in comparison to the HBD of its structural homologue, a IL-10 monomer (Figure 3.20). Amino acids with their side-chains are shown in stick form. These images with ribbon representation of α-helices were generated from the PDB structure of IL-22 (1M4R). IL-22 monomer was edited and annotated from its dimer structure (1M4R) (Nagem *Et. al.*, 2002) using the Accelyrs Discovery Studio software package.

With regards to heparin binding, IL-10 was experimentally demonstrated using surface plasmon resonance (SPR) to bind heparin/HS (Salek-Ardakani *Et. al.*, 2000). Moreover, the physiological significance of this IL-10-heparin binding was shown using IL-10-induced expression of CD-16 and CD-64 molecules on the monocyte/macrophage population within PBMCs in the presence and absence of heparin (Salek-Ardakani *Et. al.*, 2000). A closer look at the structure of the IL-10 monomer (Figure 3.19A) shows a cluster of basic residues on the carboxyl-terminal

basic peptide region (residues 99-110) which occupies the centre of the 'V'-shaped crevice. This region enriched in basic residues was very early on described as a special surface-feature on a three-dimensional structure of IL-10 (PDB ID: 1ILK) (Zdanov *Et. al.*, 1995). This sequence of basic peptide, as shown in Figure 3.19B, was proposed to promote the interaction with heparin (Salek-Ardakani *Et. al.*, 2000). Interestingly, this region has been identified as unique to IL-10 as it is conserved across different species and not shared among other members of family (Zdanov, 2010; Sabat, 2010). Very recently, the heparin-binding property of IL-10 has been further explored by predictive computational simulation methods to locate the heparin-binding site and the key residues involved in the interaction (Gehrcke & Pisabarro, 2015). Molecular docking studies predicted strong interaction of heparin chains with IL-10 via helices D (R 102, R 106, R 107) and E (N 116, K 117, K 119, K 125) (Mulloy & Forster, 2008; Gehrcke & Pisabarro, 2015). Accordingly, as seen in Figure 3.19A, the basic amino acids at the end of helix D and adjacent loop DE in IL-10 offers a concentrated surface of charged residues that is away from the core α-helical bundle (Kunze *Et. al.*, 2014). Such a charged surface seems to sterically and energetically favour the interaction with heparin chains (Samsonov *Et. al.*, 2014). These predictive results also corroborate with very recent experimental evidence by NMR citing the same GAG-binding site on IL-10 (Kunze *Et. al.*, 2016).

In case of IFN-γ, the heparin-binding property has been detected and confirmed via carboxyl-terminal basic residues (Lortat-Jacob & Grimaud, 1991a). This part of IFN-γ (which is also a regulatory peptide for its biological activity) contains a conserved stretch of basic amino acids from 128-131 residues (referred to as D1 domain: KRKR) and 137-140 residues (D2 domain: RGRR) that shows affinity for sulphate domains on heparin chains (Lortat-Jacob & Grimaud, 1991a; Vanhaverbeke *Et. al.*, 2004; Saesen *Et. al.*, 2013). However, in the available crystal structure of IFN-γ (1HIG.pdb), this carboxyl-terminal peptide (residues 124-143) is an unfolded sequence that extends away from the main structural fold and is believed to be a disordered domain, therefore highly flexible to adapt multiple conformations (Ealick *Et. al.*, 1991). The application of molecular simulation methods to proteins with such unstructured carboxyl-terminal regions enriched in basic residues may lead to artefactual results as discussed by Mulloy & Forster *(2008)*. Therefore, to date, the predictive docking calculations have not been applied to the available IFN-γ structure. Nevertheless, the mechanism of IFN-γ interaction with heparin via the C-terminal domain was recently studied by various

methods such as SPR, ITC (Isothermal Titration Calorimetry), NMR and electrostatic potential calculations. The mechanism of IFN-γ binding to heparin was identified as a two-step process with D1 domain basic residues offering strong electrostatic interactions with heparin. Whereas D2 cluster residues further stabilized and controlled the kinetics of interaction. This study thus highlighted that the two basic domains in IFN-γ contributed differently but co-operatively to induce an extended surface of positive electrostatic potential at the carboxyl-terminal domain of IFN-γ for heparin binding (Saesen *Et. al.,* 2013). Such information with molecular details for the interaction of heparin/HS with IL-10 and IFN-γ provided insights into structural features of heparin-binding domains to compare with IL-22.

Unlike in IL-10 and IFN-γ, the putative heparin-binding domain in IL-22 which is also in the carboxyl-terminal basic cluster is disrupted into smaller clusters by the acidic residues located within and those surrounding the cluster, as seen in Figure 3.20. These acidic amino acids by charge repulsion can restrict the interaction of any two, closely-spaced basic patches on the protein surface to act in concert with heparin chains. Thus, the presence of acidic residues on the surface of an IL-22 protein possibly results in an ill-defined and diffused charge distribution with weak positive electrostatic potential within the predicted HBD (Figure 3.20). This in turn may be unable to hold a strong interaction with heparin. This is also reflected in the intermolecular interaction energies obtained here by molecular docking calculations for the best-fit complex of IL-22 monomer (-809 kcal/mol) and dimer (-650 kcal/mol) with pentameric heparin chains that further supports the conclusion. The energy values for IL-22 were found to be almost twice the binding energy obtained when compared to the IL-10 dimer (-1442 kcal/mol). Therefore, the differences in energetics of binding also suggests differences in the heparin-binding capacity of IL-22 compared to other heparin-binding cytokines, IL-10 and IFN-γ, within the same family. Thus, predictive results from the docking calculations agree with experimental data presented here for IL-22.

Even though IL-10, IFN-γ and IL-22 shared secondary structural elements (Nagem *Et. al.*, 2002), one notable structural difference between them is that IL-10/IFN-γ are intercalated, physiological homodimers (Logsdon *Et. al.*, 2004; Zdanov, 2004; Nagem *Et. al.*, 2006). IL-10 and IFN-γ monomers are stabilised by formation of intertwining, 'V' shaped homodimers such that each domain of a 'V'-shaped homodimer contained the first four helices from one partner and the last two helices from the other partner

resulting in twofold symmetry in dimers (Walter & Nagabhushan, 1995; Logsdon *Et. al.*, 2004; Zdanov, 2004). On the other hand, as described earlier (Section 3.2.2.4) the IL-22 monomer is a biologically active form and the dimer is an interfacial homodimer with a compact structure and the buried interface area being twice as much of IL-10 or IFN-γ (Nagem *Et. al.*, 2002; Zdanov, 2010). Consequently, the surface basic residues on the IL-22 monomer remains partially buried in the dimer interface region. On the contrary, the surface basic clusters, particularly the heparin-binding regions on IL-10 and IFN-γ monomers are available twice, when they are in the dimer forms (Saesen *Et. al.*, 2013; Kunze *Et. al.*, 2014; Gehrcke & Pisabarro, 2015). This explains, to some extent, why in the case of the IL-22 dimer the surface basic residues are less likely to be accessible for binding to heparin. Moreover, this may also be one of the reasons for the relatively reduced affinity of the IL-22 dimer than the monomer for polyanionic chains like heparin.

Due to the above-mentioned spatial symmetry in IL-10 (Zdanov, 2004; Zdanov, 2010) and IFN-γ homodimers (Ealick *Et. al.*, 1991), this constitutes to two heparin-binding sites in these cytokine dimers. Hence, this results in strong and detectable affinity for heparin/HS chains. For instance, in the IL-10 dimer, two heparin-binding regions occur, one on either side of the central 'V'-shaped crevice at a distance of ~30-40^{o}A (Kunze *Et. al.*, 2014; Gehrcke & Pisabarro, 2015). Moreover, GAG chains of eight sugar residues or more were shown by NMR to change the IL-10-GAG binding stoichiometry suggesting that a single long GAG chain can engage both binding sites connecting the two IL-10 monomers in a IL-10 dimer (Kunze *Et. al.*, 2014; Gehrcke & Pisabarro, 2015; Kunze *Et. al.*, 2016). Whilst for HS/IFN-γ interaction, one HS molecule was proposed to bind an IFN-γ dimer such that two sulphated domains (NS), separated by an *N*-acetyl rich domain (NA) can bind directly to two IFN-γ C-termini (Lortat-Jacob *Et. al.*, 1995). Consistently, recent studies have shown that the IFN-γ dimer with the HS-binding surface shows preference to intra-HS bonds over inter-HS chain bonds (Migliorini *Et. al.*, 2015), thus supporting the previously proposed model that a single HS chain via an NA domain engages two C-termini basic clusters one on each IFN-γ monomers (Lortat-Jacob *Et. al.*, 1995; Fernandez-Botran *Et. al.*, 1999; Lortat-Jacob, 2006). Taken together, even though the structural elements (α-helices) in IL-22, IL-10, and IFN-γ are similar, the spatial distribution of basic residues along with the presence of frequent acidic residues do not seem to favour the formation of a concentrated, positively-charged HBD in IL-22 (Figure 3.20 and 3.19). Therefore,

clustering of basic residues in absence of acidic residues is important to provide optimal charge and surface complementarity for heparin/HS binding, as also recently demonstrated by Samsonov *Et. al.* (Samsonov *Et. al.*, 2014; Samsonov & Pisabarro, 2016). Hence, IL-22 lacks heparin-binding property within interferons/IL-10 family.

In summary, the current study was intended to determine whether heparin-binding is a common property among the interleukins, IL-11, IL-18 and IL-22 that share structure-function relationships with known heparin-binding family members. Combinations of various predictive and experimental approaches employed were consistent in their outcomes that this chosen group of interleukins do not bind to heparin. Thus, current findings negate the above stated hypothesis and this study further attempts to highlight the structural differences in the development of heparin-binding domains in some members and not in others of the same cytokine family. Indeed, the current findings with experimental evidence corroborates with another study published after our experimental work was completed, which used only predictive computational docking method to identify whether cytokines members of four α-helical family bind to heparin (Mulloy & Forster, 2008). More recently, NMR structural studies of a subset of Neutrophil-activating chemokines that bind to heparin/HS had a distinct GAG-binding site that were unique to each chemokine and its function, even though these chemokines shared similar structures and activate a common receptor on neutrophils (Rajarathnam *Et. al.*, 2018). Accordingly, the current finding underlines that neither heparin/HS-binding property nor the binding site is conserved among all members of cytokine family. Thus, it can be concluded from the current study that heparin binding is a characteristic of an individual interleukin and not a property that is shared by all members of a given cytokine family. However, the functional implications of this non-heparin binding property of interleukins, IL-11, IL-18 and IL-22 are discussed in Chapter 6.

CHAPTER 4

IMMUNOREGULATORY ROLE OF DERMATAN SULPHATE IN IL-12 INDUCED IFN-γ SECRETION IN MURINE NK CELLS.

4.1 Introduction

While the previous chapter studied the role of GAGs in binding to interleukins, this chapter looks into the functional role of GAGs, specifically DS. IL-12-induced IFN-γ production in NK cells is a key event in establishing Th1 type, cell-mediated immune responses. The earlier work published by Garneir *Et. al.* from our laboratory showed that among different GAG lyases used, only chondroitinase ABC inhibited IL-12-induced IFN-γ secretion indicating a role for DS in the activity of IL-12 (Garnier *Et. al.*, 2003). This chapter attempts to follow-up on these previous observations. In particular, this work has sought to elucidate the underlying mechanism by which DS affects the IL-12 signalling pathway in NK cells. To affirm this functional influence of DS, chondroitinase ABC treatment of NK cells was employed. Subsequently, methyumbelliferyl-β-D-xyloside, an inhibitor of PG biosynthesis was used to investigate the role of NK cell-surface PGs in response to IL-12.

As described earlier (Section 1.7.2, Figure 1.11) that to delineate the underlying mechanism, the proposed sites of DS-PG involvement in the IL-12 signalling pathway were tested. Firstly, to determine whether DS-PG directly affects ligand-receptor (IL-12-IL12R) binding or receptor-based activity, the phosphorylation of the IL12-specific transcription factor, STAT-4 was examined on addition of IL-12 to β-xyloside treated NK cells. Subsequently, whether DS-PG interferes with the secretion pathway of the IFN-γ protein was tested. For this, β-xyloside treated NK cells were stimulated with IL-12 and the amount of IFN-γ protein secreted into the culture supernatant was measured. Then cells were lysed to quantify IFN-γ protein levels within the cells using ELISA. Addressing these objectives has enabled us to identify whether DS-PG regulates the transcriptional, translational, or post-translational events in IL-12-induced IFN-γ expression.

4.2 Results

4.2.1 Optimisation of an *In vitro* Cellular Model for IFN-γ Secretion

The *in vitro* cellular model of NK cell activity was measured in terms of IFN-γ secreted in response to IL-12. In order to conduct this study, a dose-response curve to IL-12 in the murine NK cell line, KY-1 cells was first established. These cells were plated into

tissue culture wells and stimulated overnight with increasing concentrations of IL-12. The Culture supernatant was then tested for secreted IFN-γ by ELISA. As seen in the Figure 4.1, there is a dose-dependent increased secretion of IFN-γ by the IL-12 stimulated cells. This secretion measured against the standard curve of the IFN-γ ELISA peaked at 200 pg/ml of IL-12.

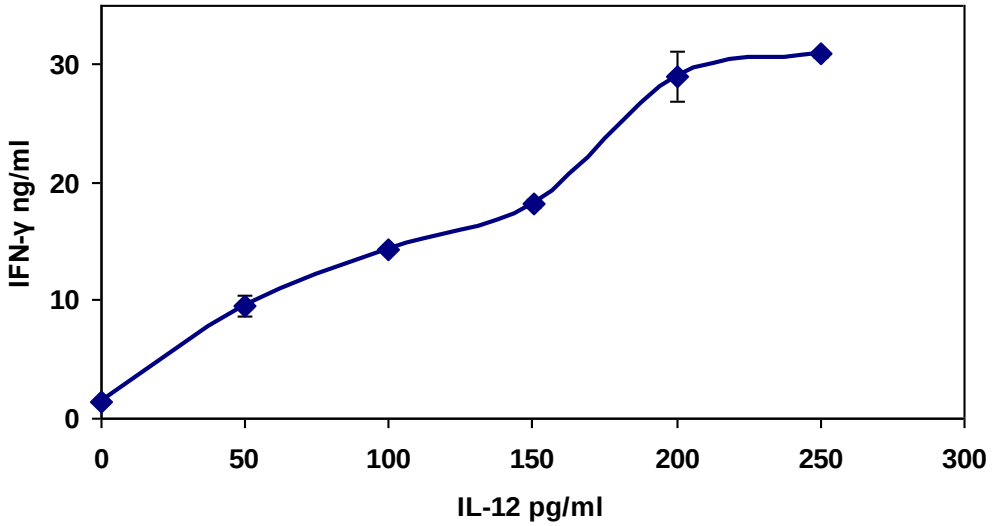


Figure 4.1: IFN-γ induction in response to increasing concentrations of IL-12: KY-1 cells (2.5 x 10^5 cells/1.7cm diameter well) primed with hIL-2 were stimulated overnight with increasing doses of IL-12, 0-250 pg/ml. Conditioned supernatants were assayed for mIFN-γ by ELISA. Results represent the mean of triplicate cultures at each point, with error bars showing ±SEM (standard error mean), not shown where smaller then symbol size. One representative experiment of three independent experiments performed is shown here.

IL-12 is known to induce NK cells and T cells to secrete high amounts of IFN-γ (Gately *Et. al.*, 1998). These results demonstrate the same with the KY-1 cell line. Since 200 pg/ml of IL-12 was found to be optimum concentration for IFN-γ secretion, the suboptimal concentration 150 pg/ml was selected for all our future experiments. This would allow us to detect either an increase or decrease in rate of IFN-γ secretion in the presence of β-xylosides or chondroitinase ABC.

4.2.2 Effect of Chondroitinase ABC on IL-12 Induced NK Cell IFN-γ Secretion

Having established that the KY-1 cells responded to exogenous IL-12 by IFN-γ secretion, the involvement of DS in NK cell produced IFN-γ was then examined using enzyme chondroitinase ABC, which strips cell-surface CS/DS. Previous work from our laboratory reported that KY-1 cells cultured in the medium containing recombinant

chondroitinase ABC (0.3 IU/ml) showed significant inhibition (30%) of IL-12 stimulated IFN-γ secretion. This effect was found to be specific to chondroitinase ABC activity, as use of other GAG lyases like heparinase I, heparinase II, hyaluronidase, and chondroitinases AC I and II did not affect IFN-γ secretion (Garnier *Et. al.*, 2003). These previous observations suggested that the DS chains are involved in the process. In the current study, initially this enzymatic approach was adopted to establish the effect of the removal of cell-surface DS on the cellular response towards IL-12. The effect of chondroitinase ABC on IL-12 bioactivity in NK cells was sought.

Prior to the addition of the enzyme, chondroitinase ABC-protease free, its activity was tested by cetylpyridinium chloride precipitation assay as described in Section 2.4.2 (data not shown). This assay detects degradation of GAG chains by the enzyme, confirming that the enzyme preparation retained its activity when diluted with the cell culture medium. Once enzyme activity under the culture conditions was confirmed, KY-1 cells stimulated with IL-12 were cultured overnight in the presence and absence of 0.1 U/ml of chondroitinase ABC. Unstimulated cells receiving no treatment served as negative controls. Secreted IFN-γ in the culture supernatant was assayed by ELISA. As seen in Figure 4.2, cells treated with the enzyme chondroitinase ABC in the presence of IL-12 showed 36% IFN-γ inhibition (bar-3), which was found to be statistically significant ($p=0.003$) against IL-12 alone stimulation (bar-2). As such, IL-12 alone treatment was taken as 100% IFN-γ secretion (bar-2) compared to negative control (bar-1). Thus, the data shown in the Figure 4.2 was comparable with the previously reported results (Garnier *Et. al.*, 2003). Indeed, this result suggests that digestion of cell surface CS/DS-GAGs of NK cells by protease-free enzyme, chondroitinase ABC reduces IL-12 induced IFN-γ secretion.

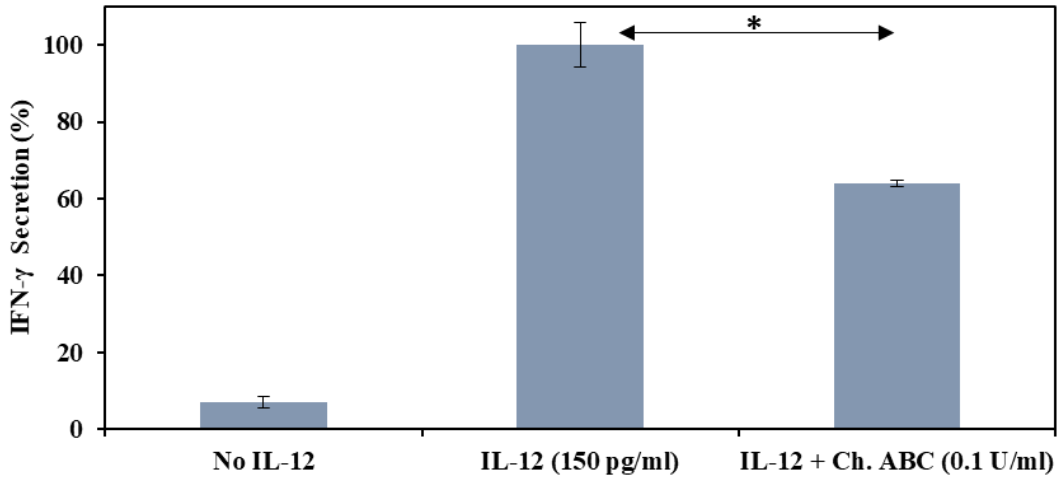


Figure 4.2: Effect of recombinant Chondroitinase ABC on IL-12 induced IFN-γ secretion: KY-1 cells (2.5 x 10^5 cells/1.7cm diameter well) were incubated overnight in the presence or absence of 150pg/ml of IL-12 and 0.1 U/ml of chondroitinase ABC. ELISA assayed the secreted mIFN-γ from the supernatant. Results are the mean of triplicate wells with error bars showing ±SEM. *Asterisk* denote $p<0.05$ for enzyme chondroitinase ABC effect in comparision to IL-12 alone stimulation for IFN-γ secretion. One representative experiment of the two independent experiments performed is shown here.

4.2.3 Effect of β-Xylosides on IL-12 Stimulated NK Cell IFN-γ Secretion

The previously assessed role of NK cell-surface DS in IL-12 stimulated IFN-γ production was further confirmed and continued using β-xylosides. As detailed earlier in Section 1.7.3.1, β-xylosides are well-established inhibitors of intact PG synthesis where they act as alternative acceptors for the enzyme galactosyltransferase in the initiation of GAG chain synthesis. Therefore, the treatment with β-xylosides results in increased synthesis and secretion of free GAG chains and decreased PG synthesis (Okayama *Et. al.*, 1973; Christmas *Et. al.*, 1988; Fritz *Et. al.*, 1994b; Kuberan *Et. al.*, 2008). Our laboratory recently implicated DS-PGs in the biological activity of IL-12 at the cellular level, in part, by finding that in the presence of 75 µM methyumbelliferyl-β-D-xyloside, IL-12 stimulated NK cells showed a reduction (by 70%) in the secretion of IFN-γ. Furthermore, the exogenous addition of soluble CS/DS or heparin as GAGs,

along with β-xyloside, could not alter this inhibitory effect on IFN-γ secretion (Garnier *Et. al.*, 2003).

The current study aimed to extend this work using methyumbelliferyl-β-D-xyloside. To reproduce the previously reported experimental results (Garnier *Et. al.*, 2003), KY-1 cells stimulated with IL-12, were cultured in the presence and absence of 75 μM methylumbelliferyl-β-D-xyloside dissolved in DMSO. Since the inhibitor-β-xyloside is hydrophobic, DMSO is the most commonly used solvent for it. Therefore, it was necessary to include a solvent control-DMSO alone in the experiment. For this, IL-12 stimulated cells were treated with the same concentration (v/v) of DMSO but without methylumbelliferyl-β-D-xyloside. This would consider the effect of DMSO itself on IL-12 signalled IFN-γ production.

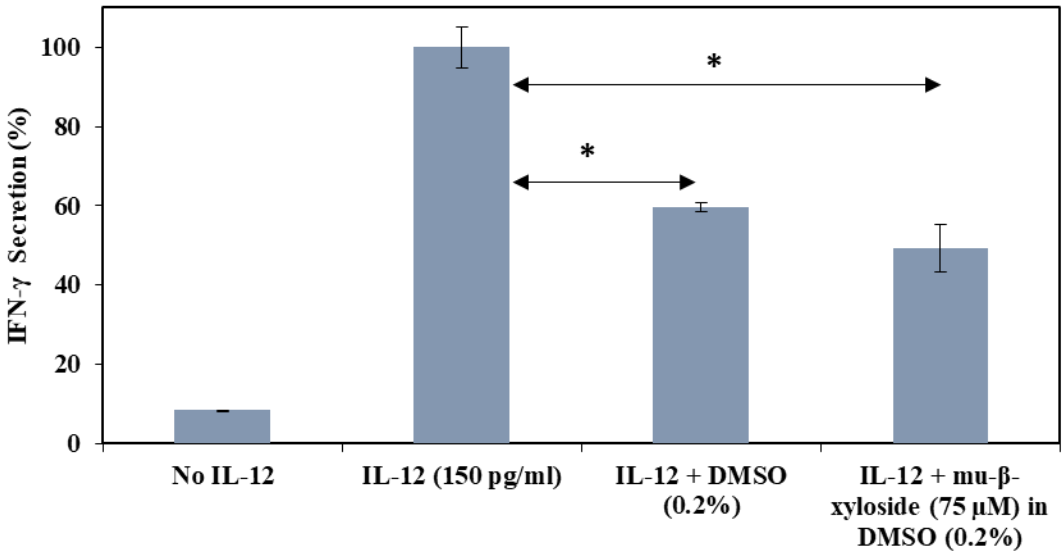


Figure 4.3: Effect of methylumbelliferyl-β-D-xyloside (mu-β-xyloside) and DMSO on IL-12 induced IFN-γ secretion: KY-1 cells (2.5 x 10^5 cells/1.7cm diameter well) were stimulated with IL-12 (150pg/ml) and cultured overnight (16 hours) in the presence and the absence of methylumbelliferyl-β-D-xyloside (75uM) in DMSO (final concentration 0.2% v/v per well). IFN-γ secreted into the supernatant medium was assayed by ELISA. Results are the mean of triplicate wells with error bars showing ±SEM. *Asterisks* denote $p<0.05$ (for both methylumbelliferyl-β-D-xyloside and DMSO alone treatments compared to IL-12 only treatment). One representative experiment of the two independent experiments performed is shown here.

As shown in Figure 4.3, unstimulated cells without treatment served as internal control (bar-1). IL-12 alone treatment (bar-2) resulted in significant IFN-γ production compared to internal control (bar-1). As such, IL-12 alone treatment was considered as 100% IFN-γ secretion. The treatment of IL-12 stimulated cells with methylumbelliferyl-β-D-xyloside (75 μM) in DMSO (bar-4) shows a significant reduction in the secretion of IFN-γ by 56% compared to IL-12 alone treatment (bar-2). This was a statistically significant reduction (p=0.044) as calculated by the non-parametric Mann-Whitney's statistical test. Further, it was found that DMSO alone (0.2% v/v) in the presence of IL-12 (bar-3) was significant in curtailing IFN-γ secretion by 44.5% (p=0.05) against IL-12 alone treatment (bar-2). This shows that the 0.2% DMSO concentration was sufficient to significantly inhibit IFN-γ induction in response to IL-12.

The effect of 75 μM methylumbelliferyl-β-D-xyloside on IFN-γ secretion (bar-4) was significant (56%, p=0.044) compared to IL-12 alone treatment (bar-2) but when compared to DMSO (0.2%) alone treatment (bar-3), the effect was not statistically significant (11%, p=0.306). This was determined by Kruskal-Wallis tests for group comparison of current experimental data (Figure 4.3). Thus, the results indicate that the inhibitory effect observed by methylumbelliferyl-β-D-xyloside in DMSO was apparently more pronounced due to effects of DMSO solvent itself. Similar effects of DMSO have been reported in other studies carried out with β-xylosides dissolved in DMSO on smooth muscle cell lines and keratinocytes (Potter-Perigo *Et. al.*, 1992). DMSO in high concentrations is toxic to the growth of cells. *p*-nitrophenyl-β-xyloside, when added at 5mM final concentration in DMSO was reported to lyse the cells, whereas the same dose of *p*-nitrophenyl-β-xyloside in PBS (phosphate buffered saline) showed no signs of cytotoxicity (Potter-Perigo *Et. al.*, 1992). Therefore, it was seen that DMSO solvent potentiated the inhibitory effect of β-xyloside on growth and cell proliferation. Hence, the use of DMSO was carefully considered in our course of experiments and their interpretations.

Nonetheless, these results (Figure 4.3) were found to differ from those previously reported (Garnier *Et. al.*, 2003), who observed 70% IFN-γ inhibition with methylumbelliferyl-β-D-xyloside (75μM) and 20% inhibition with DMSO (0.2%) solvent alone, on similar molar basis and experimental conditions. Therefore, it was thought to design and perform experiments with lower concentrations of

methylumbelliferyl-β-D-xyloside to achieve minimal interference from DMSO solvent. Moreover, as part of preliminary experiments, it was determined in the current study that methylumbelliferyl-β-D-xyloside was insoluble in ethanol and PBS. So, a stock solution of methylumbelliferyl-β-D-xyloside (1M) was prepared in DMSO. Further, experiments were conducted to determine the concentration of methylumbelliferyl-β-D-xyloside that can be added to a minimum amount of DMSO so as to get the least interference from DMSO itself. It was found that methylumbelliferyl-β-D-xyloside could be added at 60 µM and 120 µM with corresponding DMSO concentrations as 0.04% and 0.08 % v/v to the culture medium without precipitation of the inhibitor due to its lipophilic nature. Subsequently, the effect of 60 µM and 120 µM methylumbelliferyl-β-D-xyloside in DMSO on IL-12 stimulated NK cell IFN-γ secretion was tested (data not shown). The difference in the inhibitory effect exhibited by both these methylumbelliferyl-β-D-xyloside concentrations compared to their respective DMSO alone effect was to a similar extent of about 10-15%. This was a significant effect (p= 0.05) as per non-parametric Mann-Whitney's statistical test. However, this observed effect of methylumbelliferyl-β-D-xyloside was much lower compared to the reported effect by Garnier et al (Garnier *Et. al.*, 2003).

Additionally, use of another β-xyloside was thus contemplated in the current study. An analog of methylumbelliferyl-β-D-xyloside namely, *p*-nitrophenyl-β-D-xyloside, extensively known for its use as PG inhibitor in various cell culture studies was employed (Galligani *Et. al.*, 1975; Schor & Schor, 1988; Manzi *Et. al.*, 1995; Salimath *Et. al.*, 1995). Prior to the use of *p*-nitrophenyl-β-D-xyloside, its solubility in DMSO and other solvents was examined in order to achieve the required drug concentration with the minimum of added solvent. As with methylumbelliferyl-β-D-xyloside, DMSO was again the most effective solvent for *p*-nitrophenyl-β-D-xyloside.

4.2.3.1 Comparative Effects of methylumbelliferyl and p-nitrophenyl-β-D-xyloside on IL-12 Induced IFN-γ Secretion

The inhibitory effect of methylumbelliferyl-β-D-xyloside in comparison with p-nitrophenyl-β-D-xyloside on IL-12 stimulated IFN-γ secretion was assessed. KY-1 cells were stimulated with IL-12 either in the presence of 60 µM methylumbelliferyl-β-D-xyloside in DMSO or 200 µM of *p*-nitrophenyl-β-D-xyloside dissolved in DMSO.

As a solvent control in the experiment, the corresponding concentration of solvent DMSO alone (0.04% v/v) was added to the IL-12 stimulated cells. As seen in Figure 4.4, unstimulated cells without any treatment served as the internal control in the experiment (bar-1). As expected, IL-12 alone treatment (bar-2) resulted in significant IFN-γ secretion when compared to internal control (bar-1). DMSO alone treatment (bar-3) exhibited significant inhibition (by 17%, p=0.05), when compared to IL-12 alone treatment (bar-2). In addition, both 60 μM methylumbelliferyl-β-D-xyloside in DMSO (bar-4) and 200 μM of *p*-nitrophenyl-β-D-xyloside in DMSO (bar-5) were effective in reducing IFN-γ secretion by 15% and 25% respectively when compared to DMSO alone treatment (bar-3). This net inhibitory effect by methylumbelliferyl-β-D-xyloside (bar-4) and *p*-nitrophenyl-β-D-xyloside (bar-5) on IFN-γ secretion were statiscally significant (p=0.05) against DMSO alone effect (bar-3). This was analysed using non-parametric Mann-Whitney's test followed by Kruskal-Wallis test for group comparison. Thus, *p*-nitrophenyl-β-D-xyloside was observed as a more potent inhibitor of IFN-γ secretion compared to methylumbelliferyl-β-D-xyloside and consequently the more efficacious inhibitor was selected for all subsequent experiments.

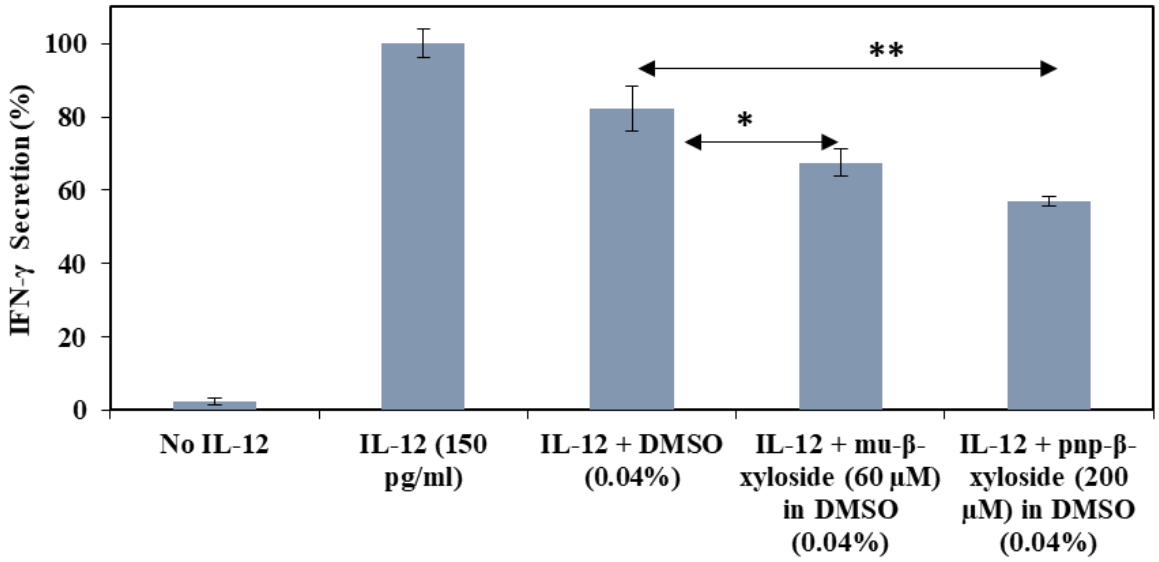
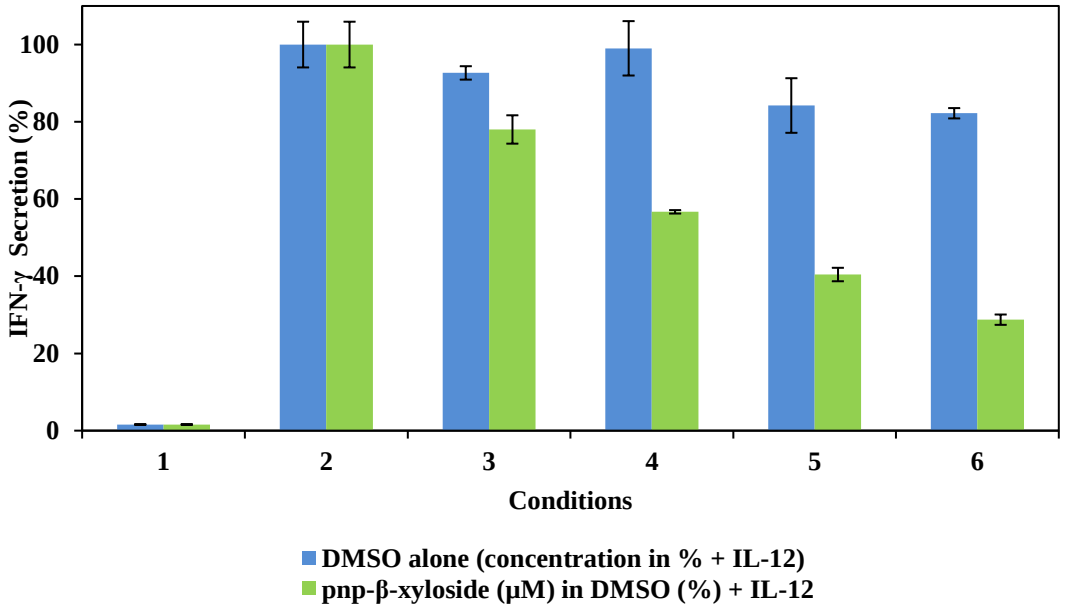


Figure 4.4: Comparative effect of methylumbelliferyl and p-nitrophenyl-β-D-xyloside on IL-12 induced IFN-γ secretion: KY-1 cells (2.5 x 10^5 cells/1.7cm diameter well) were stimulated with IL-12 (150pg/ml) and cultured overnight in the presence of methylumbelliferyl-β-D-xyloside (60µM) and p-nitrophenyl β-D-xyloside (200µM) with a relative DMSO concentration of 0.04% v/v. IFN-γ secreted in the supernatant medium was assayed by ELISA. Results are the mean of triplicate wells with error bars showing ±SEM. *Asterisks* denote $p<0.05$ for both Methylumbelliferyl-β- and p-nitrophenyl-β-xyloside in comparison DMSO alone effect. Two independent experiments were performed.

4.2.3.2 Effect of Increasing Concentrations of p-nitrophenyl-β-D-xyloside on Inhibition of IL-12 Induced IFN-γ Secretion

Next, to determine a dose dependent effect of *p*-nitrophenyl-β-D-xyloside on IFN-γ secretion, IL-12 stimulated KY-1 cells were treated with increasing concentrations of *p*-nitrophenyl-β-D-xyloside. Concentrations from 250 µM to 1500 µM of *p*-nitrophenyl-β-D-xyloside in DMSO were added. As a solvent control in the experiment, relative concentrations of DMSO alone, ranging from 0.025% to 0.15% v/v were added to IL-12 stimulated cells. After the treatment and incubation period, the culture supernatant was assayed and quantified for the levels of secreted IFN-γ protein by ELISA.



Conditions	1	2	3	4	5	6
IL-12 (150 pg/ml)	-	+	+	+	+	+
DMSO (%) ■	0	0	0.025	0.06	0.1	0.15
pnp-β-xyloside (µM) in DMSO (%) ■	0	0	250	600	1000	1500

Figure 4.5: Effect of increasing concentrations of p-nitrophenyl-β-xyloside on inhibition of IL-12 induced IFN-γ secretion: KY-1 cells (2.5 x 10^5 cells/1.7cm diameter well) were stimulated with IL-12 (150pg/ml) for 18 hours with increasing concentrations of the p-nitrophenyl-β-xyloside from 250-1500µM (■) and to the relative DMSO concentration from 0.025% to 0.15% v/v (■). The secreted IFN-γ in the supernatant was assayed using ELISA. Results represent the mean of triplicate wells at each point, with error bars showing ±SEM. One representative experiment of the two independent experiments performed is shown here.

As seen in the Figure 4.5, increasing concentrations of *p*-nitrophenyl-β-D-xyloside in the presence of IL-12 led to increased inhibition of IFN-γ secretion. Maximum inhibition of about 67% was observed for 1500 µM concentration of *p*-nitrophenyl-β-D-xyloside (green bar-6), whereas the corresponding inhibition by DMSO (0.15%) alone was reduced by 21% (blue bar-6) against IL-12 alone stimulation (blue bar-2). Concentrations above 1000 µM of *p*-nitrophenyl-β-D-xyloside or methyl-umbelliferyl xyloside has been shown in various type of cells to result in increased vacuolization or

decreased proliferative activity or both (Sobue *Et. al.*, 1987). However, in the current study, KY-1 cells exposed to concentrations higher than 600 μM of *p*-nitrophenyl-β-D-xyloside showed detachment from well surface. The detached cells were assessed for viability using nigrosin stain as described in Section 2.6. As a result, more than 50% of the detached cells were found viable under various conditions used. Nonetheless, these cells could have lost the ability to be stimulated by IL-12 and may not secrete IFN-γ. Therefore, 600 μM concentration of *p*-nitrophenyl-β-D-xyloside was considered, wherein cellular detachment was not observed but decrease in IFN-γ secretion by 50% was observed (green bar-4). Additionally, the corresponding concentration of DMSO (0.06%), in the absence of *p*-nitrophenyl-β-D-xyloside (blue bar-4), had no significant effect on IFN-γ inhibition as well as on cell detachment. Therefore, 600 μM concentration of *p*-nitrophenyl-β-D-xyloside in DMSO (0.06 %) was selected for further experimental studies to reveal the mechanism of the DS-PG effect.

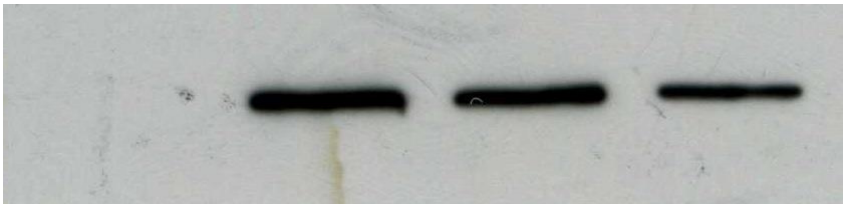
4.2.4 Effect of *p*-nitrophenyl-β-D-xyloside on IL-12 Induced Tyrosine Phosphorylation of STAT-4

Tyrosine phosphorylation of STAT-4 is a critical event associated with IL-12 receptor (IL-12R) activity and is upstream to IFN-γ expression in NK cells (Thierfelder *Et. al.*, 1996). Therefore, this event is an important control point in studying IL-12 signalling that could help determine whether DS-PG directly regulates IFN-γ expression via IL-12R activity. Having established the effect of *p*-nitrophenyl-β-D-xyloside at 600μM in 0.06% DMSO as an inhibitor of IL-12 stimulated IFN-γ secretion, the effect of this treatment on tyrosine phosphorylation of STAT-4 was examined.

KY-1 cells treated with or without *p*-nitrophenyl-β-D-xyloside were stimulated with IL-12 (150 pg/ml). Unstimulated cells receiving no treatment were used as internal control. After incubation time, cells were lysed as described in Section 2.7. As seen in Figure 4.6A, tyrosine phosphorylated STAT-4 protein was detected by western blotting using specific anti-pSTAT-4 Ab (tyr-693) at 89kDa (Figure 4.6A, Lanes 2, 3 and 4). Interestingly, unstimulated cells (Figure 4.6A, Lane 1) showed no basal levels of tyrosine phosphorylated STAT-4 and stimulation with IL-12 resulted in high levels of STAT-4 phosphorylation (Figure 4.6A, Lane 2) which was unaffected by the presence of *p*-nitrophenyl-β-D-xyloside (Figure 4.6A, Lane 4) or DMSO alone (Figure 4.6A, Lane 3). Following this, the blot (Figure 4.6A) was stripped and reprobed with anti-

STAT-4 Ab to detect the presence of total STAT-4 levels in the samples (Figure 4.6B). The intensity of bands in each lane for respective proteins was measured densitometrically using software image J, as shown in Table 4.2. Ratio of pSTAT-4 to total STAT-4 was calculated. Importantly, the results in Table 4.2 demonstrated that there was no effect of *p*-nitrophenyl-β-D-xyloside on IL-12 stimulated tyrosine phosphorylation of STAT-4.

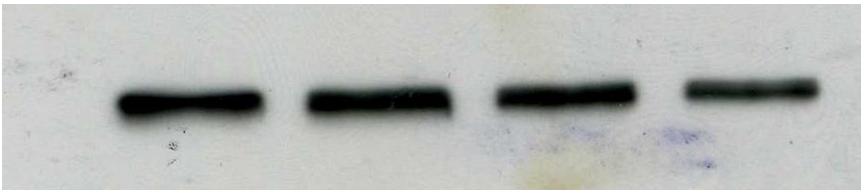
(A) WB: anti-pSTAT-4



89kDa

1 2 3 4

(B) WB: anti-STAT-4



89kDa

1 2 3 4

Lanes	1 (control)	2	3	4
IL-12 (150pg/ml)	-	+	+	+
IL-12 + DMSO (0.06%)	-	-	+	+
IL-12 + *p*-nitrophenyl-β-D-xyloside (600µM) in DMSO (0.06%)	-	-	-	+

Figure 4.6: Effect of p-nitrophenyl-β-xyloside on IL-12 induced tyrosine phosphorylation of STAT-4: KY-1 cells (2.5x 10^5 cells/ 3.4cm diameter well) cultured in the presence and absence of p-nitrophenyl β-D-xyloside (600µM) with relative DMSO concentration of 0.06% v/v for 18 hours followed by stimulation with IL-12 (150pg/ml) for 2 hours and control as unstimulated cells. Western blots were immunodeveloped with anti-pSTAT-4 (A) followed by stripping and reprobing the blot with anti-STAT-4 (B) and corresponding bands were seen. The numbers below each blot indicates cellular treatment for each lane. Densitometric analysis was performed of the blots using Image J and the ratio of pSTAT-4 / STAT-4 of the representative blot is as shown in Table 4.2. Above blot is representative of duplicate wells in an experiment. Three independent experiments were performed.

Table 4.1: Ratio of densitometric values: pSTAT-4/STAT-4

Lanes	Ratio of pSTAT-4 / STAT-4
1 No IL-12	0.0
2 IL-12 (150 pg/ml)	0.83
3 IL-12 + DMSO (0.06%)	0.85
4 IL-12 + *p*-nitrophenyl-β-D-xyloside (600μM) in DMSO (0.06%)	0.82

Based on this observation on STAT-4 phosphorylation, the result implied that *p*-nitrophenyl-β-D-xyloside did not block IL-12 binding to its membrane specific receptor, or the activation of the IL-12R. Thus, this data established that the effect of DS-PG in regulation of IL-12-induced IFN-γ secretion was independent of STAT-4 tyrosine phosphorylation. In conclusion, the involvement of DS-PG appeared to be downstream of STAT-4 phosphorylation.

4.2.5 Effect *p*-nitrophenyl-β-xyloside on IL-12 Induced Secreted and Cellular IFN-γ

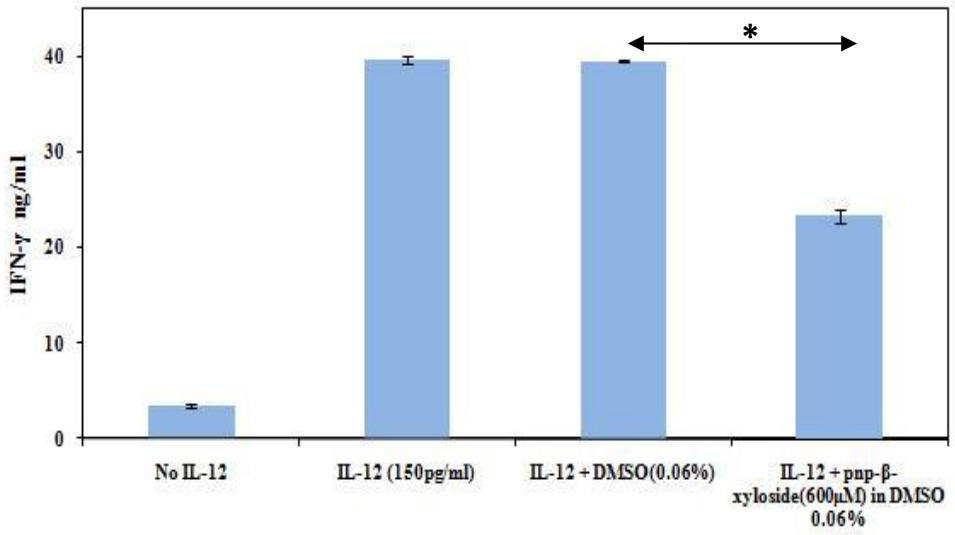
Downstream to STAT-4 activation, DS-PG could regulate at the transcriptional or translational level of IFN-γ expression. In agreement with Garnier et al's results, the current study observed partial secretion of IFN-γ in the presence of *p*-nitrophenyl-β-D-xyloside which indicated either reduced synthesis or secretion. Therefore, referring to IL-12 signalling (Figure 1.11), a possibility in this cellular investigation was to determine whether DS-PG mediates regulation through a role in the secretion of IFN-γ. For this, on stimulation with IL-12 in the presence and absence of *p*-nitrophenyl-β-D-xyloside, the levels of IFN-γ protein both within the cells and that secreted into media were quantified.

Previous reports have shown in renal visceral epithelial cells that β-xyloside caused accumulation of GAGs in the Golgi vesicles (Kanwar *Et. al.*, 1984). Moreover, IFN-γ is an inducible, secretory, glycosylated protein that follows classical ER-to-Golgi pathway of secretion. Therefore, it is plausible that IFN-γ, as a GAG-binding protein, could accumulate intracellularly, may be GAG- bound. Another report demonstrated the effect of β-xylosides on the expression of CS-PG-associated MHC class II

molecules. This study proposed a malfunctioning of Golgi apparatus that arose from the accumulation of CS within *p*-nitrophenyl-β-D-xyloside treated T cells. This resulted in altered biosynthesis and cell surface expression of MHC class II molecules and thereby disruption in antigen processing and presentation by those T cells (Rosamond *Et. al.*, 1987). Thus, it is possible that the treatment of NK cells with *p*-nitrophenyl-β-D-xyloside could result in the vesicular accumulation of IFN-γ, thereby inhibiting its secretion. Hence, it was necessary to examine the effect of *p*-nitrophenyl-β-D-xyloside on the levels of secreted and cellular IFN-γ, induced by IL-12.

KY-1 cells were stimulated with IL-12 in the presence and absence of *p*-nitrophenyl-β-D-xyloside in DMSO. Unstimulated cells without any treatment served as the internal control in the experiment. After the stimulation and incubation time, supernatants of the treated cells were removed and used to quantify secreted IFN-γ protein levels. The cells were then lysed by treatment with lysis buffer, containing 1% Triton X-100 and a cocktail of protease inhibitors. The cell lysate was used to determine cellular IFN-γ protein. So, from the same experiment, both secreted and cellular IFN-γ protein levels were quantified by ELISA. The data obtained from this experiment are shown in Figure 4.7. It is worth noting that the amount of secreted IFN-γ is expressed in ng/ml (Figure 4.7A), whereas the amount of cellular IFN-γ is in pg/ml (Figure 4.7B). As seen in the Figure 4.7A, the inhibitory effect of *p*-nitrophenyl-β-D-xyloside at 600 μM (bar-4) against effect of IL-12 alone (bar-2) or DMSO alone (0.06%) on IFN-γ sercretion (bar-3) is about 45%. Further, Figure 4.7B that shows cellular IFN-γ reveals a similar pattern and level of inhibition (45%) when compared to secreted IFN-γ in the presence of *p*-nitrophenyl-β-D-xyloside. This effect was notably a statistically significant ($p=0.05$) when compared with the effect of DMSO alone at a final concentration of 0.06% v/v. This was tested by a non-parametric Mann-Whitney's test and Kruskal-Wallis test for group comparision. Thus, the results indicate no build-up of IFN-γ on induction with IL-12 within the cell either in the presence or absence of β-xyloside. This further eliminates the possible involvement of DS-PG in debilitating secretion of IFN-γ protein. Rather, this lack of sequestration of IFN-γ within the cells implies reduced synthesis of this protein by NK cells in the presence of *p*-nitrophenyl-β-D-xyloside and therefore transcriptional regulation by DS-PG.

(A) Secreted IFN-γ



(B) Cellular IFN-γ

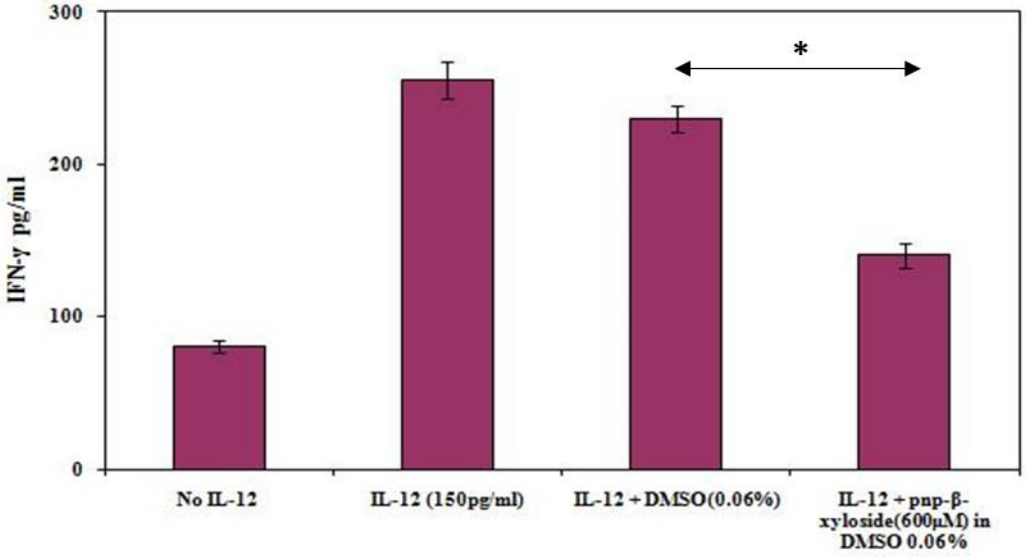


Figure 4.7: Effects of p-nitrophenyl-β-D-xylosides on IL-12 induced secreted and cellular IFN-γ:
KY-1 cells (2.5 x 10 ^{5} cells/1.7cm diameter well) were cultured for 18 hours with 600μM of p-nitrophenyl-β-xyloside, a proteoglycan inhibitor and a relative DMSO concentration as 0.06% v/v in the presence of IL-12 (150 pg/ml). The secreted IFN-γ (■) (A) in the medium and cellular IFN-γ (■) (B) under different conditions were assayed using ELISA. Results are the mean of triplicate wells with error bars showing ±SEM. *Asterisks* denote $p<0.05$ for p-nitrophenyl-β-xyloside in DMSO in comparison DMSO alone treatment at both secreted and cellular level of IFN-γ induction respectively. One representative experiment of the two independent experiments performed is shown here.

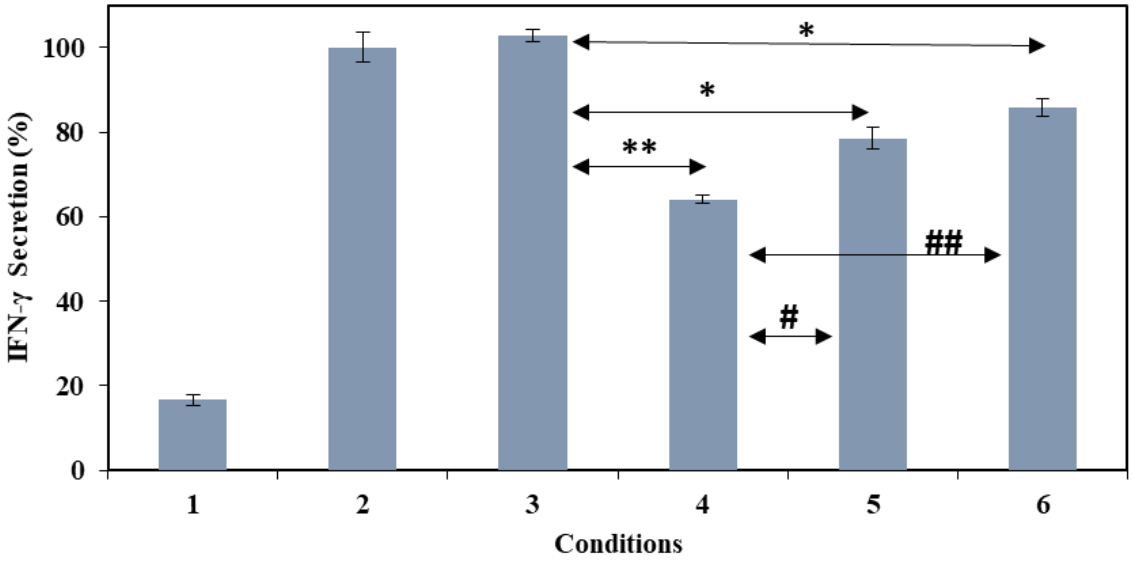
4.2.6 Evaluation of Effect of *p*-nitrophenyl-β-xyloside as a PG-inhibitor in Comparison to *p*-nitrophenyl-α-xyloside and *p*-nitrophenyl-β-galactoside on IL-12 Induced IFN-γ Secretion

Thus far, β-xylosides were used in the current set of experiments, as a tool to perturb PG synthesis in NK cells to determine the direct influence of GAGs and, by extension, PGs on IL-12 induced IFN-γ secretion. In the past, a number of studies specifically defined the role of *p*-nitrophenyl-β-D-xyloside that correlated a biological effect to PGs by using negative controls that were unable to inhibit PG synthesis (Galligani *Et. al.*, 1975; H. C. Robinson & Lindahl, 1981; Schor & Schor, 1988; Potter-Perigo *Et. al.*, 1992; Clark *Et. al.*, 2004). Therefore, it becomes important to ascertain that the observed β-xyloside inhibition of IFN-γ was due to perturbed GAG/PG synthesis, and not from other cellular or metabolic effects of β-xylosides on NK cells (Freeze *Et. al.*, 1993; Izumi *Et. al.*, 1994). To test this in the current cellular model, the effect of *p*-nitrophenyl- β-D-xyloside was evaluated by direct comparison with the effect of *p*-nitrophenyl-α-D-xyloside and *p*-nitrophenyl-β-D-galactoside on IFN-γ secretion, which served as negative controls.

p-nitrophenyl-α-D-xyloside and other β-linked sugar derivatives such as *p*-nitrophenyl-β-D-galactoside are structural analogs of *p*-nitrophenyl-β-D-xyloside (Galligani *Et. al.*, 1975; Robinson *Et. al.*, 1975). Because GAG synthesising enzymes exhibit specificity for the anomeric linkage and for the glycone structure, these exogenously added α-xylosides and β-galactosides respectively, are unable to initiate GAG synthesis and alter PG metabolism (N. B. Schwartz *Et. al.*, 1974a). Freeze *Et. al.* (1993) reported that the enzyme galactosyl transferase I (from the GAG synthetic pathway) catalyses the addition of the first Gal unit to both *p*-nitrophenyl-α-D-xyloside and *p*-nitrophenyl-β-D-xyloside in CHO (Chinese Hamster Ovarian) cells. However, the subsequent enzyme, galactosyl transferase II distinguishes between the α and β configuration of the terminal xylose, where addition of second Gal residue does not occur in the α-linked xyloside. On the other hand, *p*-nitrophenyl-β-D-galactosides are ineffective in the synthesis of free GAG chains because of specificity in substrate recognition shown by enzyme galactosyl transferase I that uses only an acceptor that has a terminal D-xylose (N. B. Schwartz *Et. al.*, 1974a; Robinson *Et. al.*, 1975). Thus, the ineffectiveness of these structural analogs of *p*-nitrophenyl-β-D-xyloside in inhibition of PG synthesis

and in concurrent stimulation of free GAG chains makes them ideal candidates as negative controls.

Further, KY-1 cells were stimulated with IL-12 in the presence and absence of the same concentration of different glycosides, namely; *p*-nitrophenyl-β-D-xyloside, *p*-nitrophenyl-α-xyloside, and *p*-nitrophenyl-β-galactoside. The solvent used for these glycosides was DMSO, added at the final concentration of 0.06% v/v. As seen in Figure 4.8, DMSO alone treatment (bar-3) was ineffective in suppressing IL-12-induced IFN-γ secretion. As expected, *p*-nitrophenyl-β-D-xyloside showed significant inhibition by 36% ($p = 0.05$) of IFN-γ secretion (bar-4), however *p*-nitrophenyl-α-xyloside (bar-5) and *p*-nitrophenyl-β-galactoside (bar-6) also exhibited IFN-γ inhibition by 21.4% and 14% respectively in comparison to DMSO alone effect (bar-3). Thus, as observed, IFN-γ inhibition by all three glycosides employed (bars 4-6) was statistically significant ($p=0.05$) against IL-12 alone stimulation (bar-2) as well as DMSO alone (bar-3) treatment. Therefore, even though *p*-nitrophenyl-β-xyloside inhibited 36% of IL-12-stimulated IFN-γ secretion but when compared to negative controls like α-xyloside (bar-5) and β-galactoside (bar-6), its net effect on IFN-γ inhibition (bar-4) was evaluated to be about 15-20%. Moreover, this difference in the effect between *p*-nitrophenyl-β-xyloside (bar-4) and the negative controls (bars-5, 6) was found statistically significant ($p=0.05$), using Kruskal-Wallis test for group comparision. Hence, the results indicate that 15-20% of the overall 36% IFN-γ inhibition by *p*-nitrophenyl-β-xyloside was due to perturbation in GAG and PG synthesis.



Conditions	1	2	3	4	5	6
IL-12 (150pg/ml)	-	+	+	+	+	+
DMSO (0.06%)	-	-	+	+	+	+
p-nitrophenyl-β-D-xyloside (600µM) in DMSO	-	-	-	+	-	-
p-nitrophenyl-α-D-xyloside (600µM) in DMSO	-	-	-	-	+	-
p-nitrophenyl-β-D-galactoside (600µM) in DMSO	-	-	-	-	-	+

Figure 4.8: Effects of α and β xylosides on secreted IFN-γ:
KY-1 cells (2.5 x 10^5 cells/1.7cm diameter well) were cultured for 18 hours each with 600 µM equivalent of 0.06% v/v DMSO with p-nitrophenyl-β-xyloside, a proteoglycan inhibitor and p-nitrophenyl-α- xyloside and p-nitrophenyl-β- galactoside as negative controls respectively in the presence of IL-12 (150 pg/ml). The secreted IFN-γ (■) in the medium under different conditions was assayed using ELISA. Results are the mean of triplicate wells with error bars showing ±SEM.
***** denotes $p<0.05$ for all three glycosides in comparision to DMSO alone treatment.
# denotes $p<0.05$ for p-nitrophenyl-β-xyloside in comparision to p-nitrophenyl-α-xyloside and p-nitrophenyl-β-galactoside effect respectively on IFN-γ secretion. One representative experiment of the two independent experiments performed is shown here.

4.3 Discussion

The production of IFN-γ from NK cells is an *in vitro* model representing initiation of Th1 responses. The current study showed that NK cell-surface DS-PGs were involved in IL-12-induced IFN-γ production. Thus, the current findings support the concept that GAGs can modulate immune responses. Concurrent with previous observations by Garnier *Et. al.* (2003), the current study removed cell surface CS/DS-PGs from NK cells using the enzyme chondroitinase ABC. This reduced the cellular response to IL-12 stimulation and resulted in decreased IFN-γ secretion by ~36%. These results confirm the previous finding from Garnier *Et. al.*, however its not known as to how the NK cell-surface DS-PG might affect the bioactivity of IL-12 to produce IFN-γ. The goal of the current study was therefore to elucidate the mechanism of DS-PG involvement in IL-12 signalling-dependent IFN-γ secretion, using a PG inhibitor, β-xyloside.

The current study has established the functional dependence of IL-12 on DS-PG in the production of IFN-γ using *p*-nitrophenyl-β-xyloside. Importantly, the current study attempts to highlight the level of cellular regulation at which IFN-γ expression is dependent on DS-PG. However, in understanding this regulatory mechanism, various sites in the IL-12-activated signalling pathway in IFN-γ production were proposed as points of possible DS-PG involvement (Section 1.7.2, Figure 1.11). Each proposed site was tested systematically for its validation in the current study. The first potential site hypothesised for DS-PG involvement was in cross-bridging the ligand-receptor (IL-12-IL-12R) interaction, similar to the role of heparin in initiating FGF-FGFR interaction and signalling (Yayon *Et. al.*, 1991). This was probed by tyrosine phosphorylation of STAT-4, which is a key event in IL-12 signalling and brought about by activated IL-12 receptor-associated, JAK2 and TYK2 kinases. In this context, the current finding demonstrated that treatment of NK cells with *p*-nitrophenyl-β-xyloside did not alter IL-12 stimulated tyrosine phosphorylation of STAT-4 (Figure 4.6). This indicated that the role of DS-PG in IL-12 signalling is not mediated through the transcription factor, STAT-4. Hence, this eliminated the first possibility of DS-PG involvement in IL-12-IL-12R interactions and therefore, in receptor-associated JAK activity. Moreover, tyrosine phosphorylation of STAT-4 was shown to control its DNA-binding activity (Bacon *Et. al.*, 1995; S. S. Cho *Et. al.*, 1996), this further suggests that downstream to

STAT-4 phosphorylation, STAT-4-DNA binding activity was unlikely to be regulated by DS-PG.

Further, the results demonstrated significantly lowered amounts of IFN-γ protein in the culture supernatants as well as in the cell lysates on addition of IL-12 to *p*-nitrophenyl-β-D-xyloside treated NK cells (Figure 4.7). Interestingly, the level of inhibition on the intracellular and extracellular concentrations of IFN-γ protein was same. This data clearly showed that the IFN-γ protein was not being sequestered intracellularly but was rather synthesised in reduced amounts in cells to have been secreted in lower quantities, extracellularly. Importantly, this data suggested that the mechanism regulating IFN-γ expression by DS-PG is not mediated through interference with the IFN-γ secretory pathway. In addition, this experimental step which determined IFN-γ protein release, rules-in the second possibility of transcriptional regulation and rules-out the third possibility of translational or post-translational blockage by DS-PG. Thus, the current findings validated the second potential site that was downstream of STAT-4 phosphorylation at the transcriptional level in IL-12 signalling. However, to ascertain this finding, inhibitors of protein-synthesis at the translational level such as cycloheximide or puromycin, in the presence and absence of *p*-nitrophenyl-β-D-xyloside can be used in future experiments. Further, to evaluate the role of DS-PG in the transcriptional regulation of IFN-γ expression, it would provide important experimental data to determine and quantify IL-12-induced IFN-γ mRNA levels in *p*-nitrophenyl-β-D-xyloside or chondroitinase ABC treated NK cells by a ribonuclease protection assay or RT-PCR in future studies. Consequently, the current findings conclude that NK cell-surface DS-PG modulates the transcriptional process of IL-12-induced IFN-γ synthesis. This conclusion is in concurrence with the recent findings from a study which demonstrated that decorin, a DS-PG affects IFN-γ expression at transcriptional level. This was confirmed using decorin KO mouse which showed significantly reduced IFN-γ mRNA expression and protein levels in response to an allergy-induced inflammation (Bocian *Et. al.*, 2013).

Based on the results obtained in the current study, these imply that the transcriptional suppression of IFN-γ production by DS-PG is independent of the IL-12-activated JAK-STAT-pathway. Consistent with this finding, DS was shown to have no effect on STAT-signalling induced ICAM-1 expression. However, DS through an unknown mechanism activates an alternate, NF-κB mediated signalling pathway in the increased

transcription and expression of ICAM-1 in endothelial cells (Penc *Et. al.*, 1999). Besides the well-studied JAK-STAT pathway, IL-12 is also known to activate the p38 MAPK pathway in the transcriptional regulation of IFN-γ production and hence Th1 differentiation (Zhang & Kaplan, 2000; Watford *Et. al.*, 2004). This suggests the existence of an alternative mechanism in IFN-γ regulation and DS-PG might regulate through this mechanism. p38 MAPK signalling phosphorylates IL-12-activated STAT-4 on specific serine residues. In addition to tyrosine phosphorylation, serine phosphorylation of STAT-4 is essential to IFN-γ production and is required for maximal transcriptional activity of STAT-4 (Morinobu *Et. al.*, 2002). Thus, alteration in serine phosphorylation of STAT-4 could be one potential mode of transcriptional regulation by DS-PG via the p38 MAPK pathway.

Additionally, independent of STAT-4, the direct implications of IL-12-activated p38 MAPK pathway on IFN-γ production has been documented in NK and T cells. This was evident from reduced IFN-γ secretion by STAT-4-deficient Th1 cells in the presence of the p38 MAPK inhibitor-SB203580 (Zhang & Kaplan, 2000). In the absence of STAT-4, p38 MAPK is proposed to act via ATF-2 (Activating Transcription Factor 2), a member of AP-1 family of transcriptional activators (Zhang & Kaplan, 2000). In this context, Rajgopal *Et. al.* (2006) reported that the mechanism by which heparin enhances IL-11-induced STAT-3 activation was independent of serine phosphorylation of STAT-3. However, the mechanism involved was direct up-regulation of MAPK pathway. This was shown in osteoclast formation of murine calvaria cells. This study also showed that heparin augmented IL-11-induced gene expression of RANKL (Receptor Activator of NF-κB Ligand), an essential element in *in vitro* osteoclast formation. Further, the expression of RANKL was suggested via activation of the MAPK/ERK pathway (Rajgopal *Et. al.*, 2006). Similarly, another study demonstrated the role of DS via p38 MAPK signalling pathway in the regulation of osteoclast formation and bone metabolism. The presence of DS with RANKL was shown to strongly reduce phosphorylation of p38 MAPK and ERK (Extracellular signal-Regulated Kinase) in murine monocytic cells than in cells stimulated with RANKL alone (Shinmyouzu *Et. al.*, 2007). Collectively, these findings suggest that DS chains may regulate the p38 MAPK pathway, a plausible mechanism through which DS-PG can regulate IL-12-stimulated IFN-γ expression. However, this hypothesis requires further investigation.

During the current investigation, the specificity of β-xyloside in the inhibition of IFN-γ production as a result of disrupted PG synthesis was also determined. However, it is important to note that this aspect of study was not examined in the previous work by Garnier *Et. al.* (2003). In the current study, this was demonstrated by comparing the effect of *p*-nitrophenyl-β-xyloside with the effects of *p*-nitrophenyl-α-xyloside and *p*-nitrophenyl-β-galactoside respectively, on IFN-γ production. As explained earlier in Section 4.2.6, *p*-nitrophenyl-α-xyloside cannot initiate GAG chain synthesis (Canfield *Et. al.*, 1994) due to structural anomerism whilst *p*-nitrophenyl-β-galactoside is ineffective in initiation of GAG synthesis (Bradbeer *Et. al.*, 1990) due to glycone substrate specificity by enzymes in PG synthesis. Therefore, both these glycosides are ineffective in disrupting PG synthesis. Unexpectedly, it was observed that *p*-nitrophenyl-α-xyloside and *p*-nitrophenyl-β-galactoside also inhibited IFN-γ secretion in NK cells, albeit to a lesser extent, when compared to *p*-nitrophenyl-β-xyloside. Thus, it was concluded that IFN-γ inhibition (35-45%) observed in *p*-nitrophenyl-β-xyloside treated NK cells was only partially mediated (15-20%) by disruption in PG synthesis. Further, the data suggests that, to varying extents, addition of any xyloside, whether α- or β-, weakens NK cell responses to IL-12 in terms of IFN-γ secretion. Hence, it implies that *p*-nitrophenyl-β-xyloside perhaps exerted other cellular effects, besides altering PG synthesis, which are shared with *p*-nitrophenyl-α-xyloside, as previously reported in number of other studies (Potter-Perigo *Et. al.*, 1992; Freeze *Et. al.*, 1993; Canfield *Et. al.*, 1994).

Even though, the mechanism(s) by which *p*-nitrophenyl-α-xyloside and *p*-nitrophenyl-β-xyloside could have exerted cellular and physiological effects in NK cells are not known, few possibilities could be proposed based on their effects on other cell types and their metabolism. For example, both *p*-nitrophenyl-α-xyloside and *p*-nitrophenyl-β-xyloside inhibit proliferation of several types of cells, independent of their effects on GAG/PG synthesis (Potter-Perigo *Et. al.*, 1992). Similarly, *p*-nitrophenyl-α-xyloside is a comparable inhibitor of glycolipid synthesis to *p*-nitrophenyl-β-xyloside in variety of cells, such as macrophage-like cells, CHO (Chinese Hamster Ovarian) cells, and melanoma cells (Freeze *Et. al.*, 1993). Both α- and β-xylosides are also known to inhibit protein synthesis however, this effect was proposed to be secondary to glycolipid synthesis (Canfield *Et. al.*, 1994). Thus, it has been well established that β-xyloside has other metabolic effects besides altering PG synthesis, which apparently also surfaced during this study. This meant that the inhibition of IFN-γ production

resulting from β-xyloside treatment was most likely, partially due to some of these other cellular effects. Indeed, this brought a limitation to the current mechanistic study. Unfortunately, due to comparable generalised effects of *p*-nitrophenyl-β-xyloside relative to the contribution of GAGs/PGs as revealed in current investigation, it was not possible to continue this study on the functional role of DS-PG in IFN-γ expression without addressing this limitation (as discussed in Chapter 6).

CHAPTER 5

IMMUNOREGULATORY ROLE OF TGF-β1 IN IL-12 INDUCED IFN-γ SECRETION IN MURINE NK CELLS

5.1 Introduction

From the previous chapter, it was seen that KY-1 cells were demonstrated to be a reliable *in vitro* model for the study of IL-12-induced IFN-γ production and regulation. Given the critical role of IL-12 and its effector cytokine, IFN-γ, in determining the nature and outcome of immune responses, particularly during infectious diseases and tumour development, understanding the regulation of IL-12 signalling in IFN-γ production is of major interest. As described earlier, in Section 1.8.1, TGF-β1 is an anti-inflammatory and immunosuppressive cytokine candidate and has been well reported to inhibit IL-12 induced NK and T cell proliferation, IFN-γ production, cytotoxic activity, and Th1 differentiation (Bellone *Et. al.*, 1995; Laouar *Et. al.*, 2005; J. T. Lin *Et. al.*, 2005; Lewis *Et. al.*, 2015). Therefore, the role of TGF-β1 was central to number of studies investigating the crosstalk with IL-12 signalling and IFN-γ production (Bright & Sriram, 1998; Pardoux *Et. al.*, 1999; Sudarshan *Et. al.*, 1999; Yu *Et. al.*, 2006), however the mechanistic understanding of IFN-γ suppression by TGF-β1 in NK cells is not well understood at the molecular level.

The aim of this chapter is to systematically study the molecular events underlying the suppressive effects of TGF-β1 on IL-12-mediated IFN-γ production using KY-1 cells. Firstly, the anti-proliferative activity of TGF-β1 was investigated as a possible mechanism in the inhibition of IFN-γ production. This was followed by examining the effects of TGF-β1 on the components of IL-12 signalling such as tyrosine and serine phosphorylation of STAT-4. In addition to STAT-4, T-bet is another significant transcription factor in mediating IFN-γ gene expression (Szabo *Et. al.*, 2000; J. Y. Cho *Et. al.*, 2003). Therefore, it was important to determine whether T-bet is a critical mediator of TGF-β1 suppressed IFN-γ production. Further investigation was made using reporter gene assays in the convergence of IL-12 and TGF-β1 signalling pathways that regulated IFN-γ gene expression. The effect of TGF-β1 on IFN-γ promoter activity was determined using transiently transfected KY-1 cells with an IFN-γ promoter construct expressing luciferase gene.

5.2 Results

5.2.1 Effect of TGF-β1 Induced Suppression of IFN-γ Secretion in IL-12 Stimulated murine NK cells

To assess the effect of TGF-β1 induced IFN-γ suppression, KY-1 cells were pre-treated with increasing concentrations of TGF-β1 (5, 10 and 15ng/ml) for 30 mins. This was followed by stimulation with 10ng/ml of IL-12 for 24 hrs. The cell free supernatant was assayed for IFN-γ using ELISA.

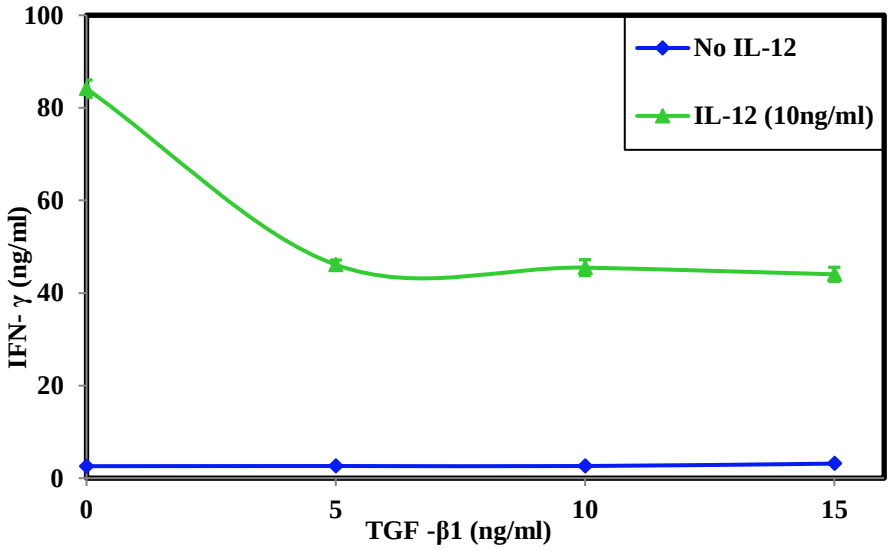


Figure 5.1: Effect of TGF-β1 on IL-12 induced IFN-γ secretion: KY-1 cells (2.5 x 10^5 cells/1.7 cm diameter well) were pre-incubated for 30 mins with increasing concentrations of TGF-β1 (5, 10 or 15 ng/ml) followed by addition of IL-12 (10 ng/ml) and cultured for 24 hrs. The secreted IFN-γ in the supernatant was assayed using ELISA. Results represent the mean of duplicate wells at each point with error bars showing ± SD, not seen where smaller than symbol size. One representative experiment of two independent experiments performed is shown here.

As seen in Figure 5.1, in the presence of TGF-β1 (irrespective of its concentration used 5, 10 or 15 ng/ml), IL-12-stimulated IFN-γ production was suppressed by about 50%. This suppressive effect of TGF-β1 was found to be statistically significant as indicated by $p<0.05$ (ANOVA). However, the presence of TGF-β1 alone had no effect on IFN-γ production. Based on these observations, a concentration of 10 ng/ml of TGF-β1 was selected for further experimental work, which was also in accordance with the literature

(Sudarshan *Et. al.,* 1999). Thus, this data demonstrates partial suppression of IL-12-stimulated IFN-γ production by TGF-β1.

5.2.2 Effect of TGF-β1 on IL-12 Stimulated NK Cell Proliferation and IFN-γ Secretion

TGF-β1 has been reported in T cells to inhibit IL-12-stimulated cell proliferation, survival and IFN-γ production (Bright & Sriram, 1998). Similarly, studies have demonstrated that TGF-β1 suppressed NK cell proliferation, cell cycle progression and concurrently impaired production of cytokines, including IFN-γ in response to IL-12/IL-15 cytokine stimulations (Bellone *Et. al.,* 1995; Wilson *Et. al.,* 2011) and during viral infections (Su *Et. al.,* 1991). These studies suggested that the anti-proliferative effect of TGF-β1, partly, formed the basis for decreased IFN-γ production from these cells. Therefore, this raised a question whether the observed reduction in IFN-γ production (Figure 5.1) in the current study was a secondary effect to inhibition of cell proliferation by TGF-β1. To elucidate this possibility, the total cell numbers were counted in the presence and absence of TGF-β1 along with the measurement of secreted IFN-γ in the culture supernatant by ELISA, at various time points.

KY-1 cells (3×10^4 cells/well) treated with and without TGF-β1 (10 ng/ml) for 30 mins were plated for 6 hours, 16 hours, and 24 hours with IL-12 stimulation (10 ng/ml). After the defined period of incubation with IL-12, secreted IFN-γ in the supernatant media was detected by ELISA and the cells were counted for viability using nigrosin stain (as described in section 2.6). Figure 5.2 shows the time course of IFN-γ secretion in the presence of TGF-β1, which by 6 hours was reduced by approximately 50% ($p<0.001$). Similarly, a significant level of inhibition was observed at 16 hours (by 41.91%, $p<0.001$) and 24 hours (by 61.23%, $p<0.001$), which confirmed the suppressive effects of TGF-β1 on IFN-γ secretion at multiple time points.

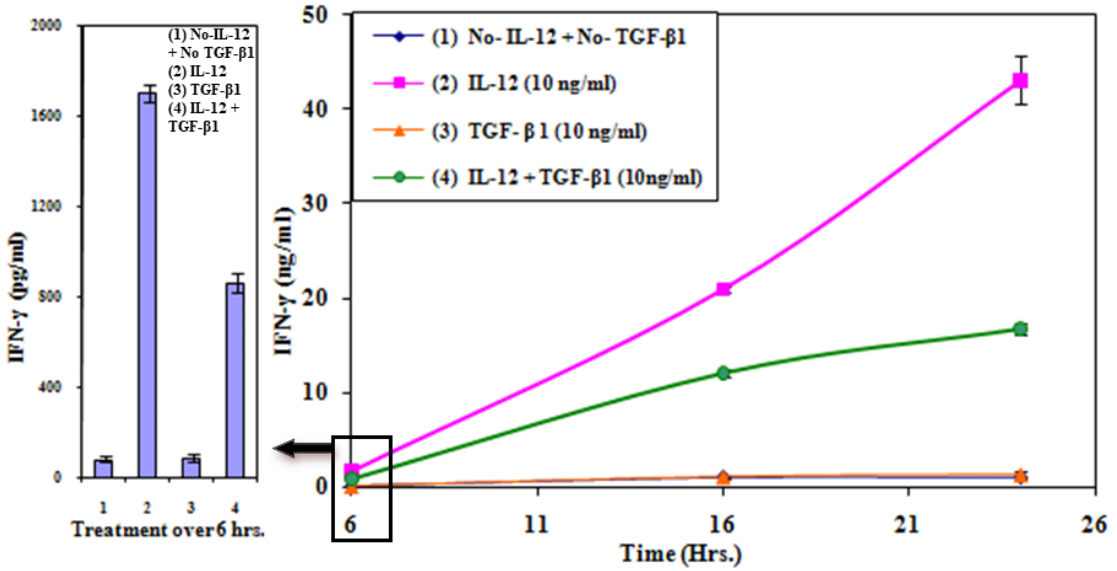


Figure 5.2: Time course of IFN-γ secretion in the presence of TGF-β1: KY-1 cells (3 x 10^4 cells / 0.8cm diameter well) were pre-incubated for 30 mins. with or without TGF-β1 (10 ng/ml) followed by addition of IL-12 (10 ng/ml) and cultured for 6 hrs, 16 hrs and 24 hrs, after which they were counted for viability using Nigrosin stain as shown in the result Table 5.1. The secreted IFN-γ was assayed using ELISA. The left-hand panel shows IFN-γ secreted at 6 hrs on expanded axis. Results represent the mean of triplicate wells at each point with error bars showing ± SEM, not seen where smaller than symbol size. One representative experiment of three independent experiments performed is shown here.

Using the same culture, the effect of TGF-β1 on cell proliferation in the presence of IL-12 was analysed by cell counting and cell viability of attached and detached cells at multiple time points of 6, 16 and 24 hours. As seen in Table 5.1, at 6 hours of stimulation with IL-12, in the absence and presence of TGF-β1 (rows 2 vs 4), there was no significant difference in the total cell numbers (column-Ta+d) and % cell viability (column-% Va+d). Yet, IL-12 stimulated IFN-γ production was reduced by 50% in the presence of TGF-β1 at 6 hours (column-IFN-γ, rows-2 vs 4). This indicated that TGF-β1 rapidly inhibited IFN-γ production without affecting cell numbers and viability.

Similar inference can be drawn from the data at 16 and 24 hours that the presence of TGF-β1 did not affect cell proliferation (column- Ta+d, rows 6 vs 8, rows 10 vs 12) and more importantly, the cell viability (column- %Va+d, rows 6 vs 8, rows 10 vs 12). Nevertheless, as time progressed to 16 hours, there was significant increase in the total number of cells (column-Ta+d, rows-5 to 8) indicating cell proliferation. This

proliferative growth was seen under all conditions and was not selective to IL-12 stimulation. After 24 hours, there was a minimal or no increase in total cell numbers (column-Ta+d, rows-9 to 12) to that observed at 16 hours under all cytokine treatments. Rather, cell viability decreased significantly over the time until 24 hours (column- V a+d). One possible reason could be that at both 16 and 24 hours, significant number of cells were found detached irrespective of any cytokine treatment as compared to 6 hours. This was a concern as KY-1 cells are mostly adherent cells and significant cell detachment may indicate cell death. Therefore, the detached cells from 16- and 24-hour plate were assayed for cell viability (column- %Vd, rows 5 to 12), and only 25-40% of cells were found viable. This demonstrated that most detached cells were dead; while others that remained viable, might arguably have reduced protein synthesis or secretion. Thus, cell detachment and death observed was higher after 24 hours than at 16 hours. Although, cell density per well was proportionately considered to the surface area of the well for this assay, the reason for cell detachment on longer hours of incubation was not clearly understood.. Importantly, the results from the time points of 16 hours and 24 hours indicated that IL-12 did not induce cell proliferation, as seen in Table 5.1 (column Ta+d, rows 5 vs 6 and rows 9 vs 10). Additionally, TGF-β1 treatment alone or in the presence of IL-12 did not result in decreased cell numbers (column Ta+d, rows 6 vs 8, rows 10 vs 12), despite a reduction in IFN-γ production on IL-12 stimulation (column- IFN-γ, rows 6 vs 8, rows 10 vs 12). Hence, the data suggests that the IFN-γ inhibition by TGF-β1 was independent of its effect on cell cycle progression and anti-proliferative effect.

Time (hrs)	Rows	Cytokines	Attached Cells x 10^4 per well ± SEM					Detached Cells x 10^4 per well ± SEM					Total Cells x 10^4 per well ± SEM				
			Va	NVa	Ta	% Va	*P* value	Vd	NVd	Td	% Vd	*P* value	Ta+d	% Va+d	*P* value	IFN-γ (ng/ml)	*P* value
6	1	No IL-12 + No TGF-β1	2.90± 0.15	0.23± 0.03	3.13± 0.14	93	1.0	0.78± 0.03	0.14± 0.0	0.92± 0.03	85	0.415	4.05± 0.18	91	0.920	0.08± 0.01	0.0001
	2	IL-12 (10 ng/ml)	2.30± 0.2	0.20± 0.06	2.50± 0.24	92	-	0.90± 0.08	0.07± 0.0	0.97± 0.12	93	-	3.47± 0.26	92	-	1.74± 0.03	-
	3	TGF-β1 (10 ng/ml)	2.47± 0.03	0.30± 0.1	2.77± 0.13	89	0.83	0.62± 0.06	0.12± 0.02	0.74± 0.05	84	0.325	3.50± 0.11	88	0.477	0.08± 0.01	0.0001
	4	IL-12 + TGF-β1	2.50± 0.28	0.33± 0.03	2.83± 0.26	88	0.55	0.52± 0.10	0.07± 0.04	0.59± 0.13	88	0.846	3.42± 0.30	88	0.358	0.86± 0.04	0.0001
16	5	No IL-12 + No TGF-β1	2.70± 0.29	0.23± 0.06	2.93± 0.33	92	0.39	0.73± 0.15	1.87± 0.24	2.60± 0.10	28	0.945	5.53± 0.24	62	0.807	1± 0.23	0.0001
	6	IL-12 (10 ng/ml)	2.17± 0.08	0.37± 0.03	2.53± 0.08	86	-	0.95± 0.04	2.20± 0.38	3.15± 0.40	30	-	5.83± 0.32	56	-	21 ± 0.3	-
	7	TGF-β1 (10 ng/ml)	2.73± 0.29	0.23± 0.09	2.97± 0.34	92	0.37	1.57± 0.63	2.47± 0.32	4.04± 0.94	39	0.998	6.97± 1.01	61	0.879	1.05± 0.05	0.0001
	8	IL-12 + TGF-β1	1.50± 0.11	0.23± 0.09	1.73± 0.11	87	0.99	0.87± 0.37	2.47± 0.15	3.34± 0.41	26	0.744	5.07± 0.42	47	0.389	12.2± 0.46	0.0001
24	9	No IL-12 + No TGF-β1	1.20± 0.15	0.57± 0.17	1.77± 0.03	68	0.93	0.93± 0.09	3.13± 0.07	4.06± 0.12	23	0.142	5.83± 0.09	37	0.311	1.19± 0.09	0.0001
	10	IL-12 (10 ng/ml)	1.20± 0.11	0.40± 0.17	1.60± 0.24	75	-	1.25± 0.04	3.10± 0.25	4.35± 0.32	28	-	6.03± 0.50	42	-	43.3± 2.5	-
	11	TGF-β1 (10 ng/ml)	0.80± 0.0	0.30± 0.10	1.10± 0.10	73	0.99	1.27± 0.09	3.17± 0.38	4.44± 0.38	29	0.968	5.53± 0.44	37	0.459	1.36± 0.36	0.0001
	12	IL-12 + TGF-β1	0.50± 0.1	0.37± 0.14	0.87± 0.06	58	0.63	1.7± 0.06	2.9± 0.15	4.60± 0.12	37	0.193	5.47± 0.13	40	0.865	16.79± 0.57	0.0001

Table 5.1: Cell count, cell viability and IFN- γ secretion in the presence and absence of TGF-β1 by IL-12 stimulated NK cells at 6, 16 and 24 hrs.
Please refer to the legend for this table on next page.

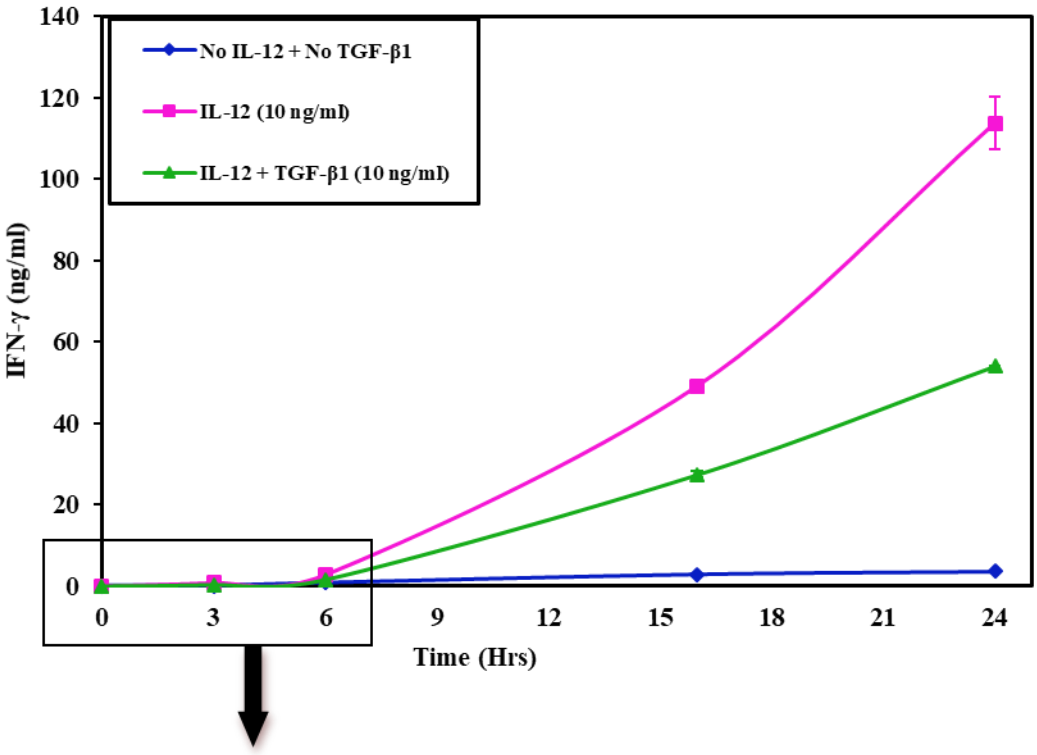
Table 5.1: Cell count, cell viability and IFN- γ secretion in the presence and absence of TGF-β1 by IL-12 stimulated NK cells at 6, 16 and 24 hrs: KY-1 cells (3 x 10^4 cells / 0.8cm diameter well) were pre-incubated for 30 mins. with or without TGF-β1 (10 ng/ml) followed by addition of IL-12 (10 ng/ml) and cultured for 6 hrs, 16 hrs and 24 hrs. The secreted IFN-γ was assayed using ELISA. Both attached and detached cells were counted for cell viability as shown above in columns, V**a**= attached viable cells, NV**a**= attached non-viable cells, T**a** =V**a** + NV**a** (Total attached cells), %Va = % viability of attached cells, V**d**= detached viable cells, NV**d**= detached non-viable cells, T**d**= V**d**+NV**d** (Total detached cells), %Vd = % viability of detached cells T**a+d** = Total attached and detached cells, %Va+d = % viability of attached and detached cells. Rows = each row represents particular experimental condition. Results are mean of triplicate wells per condition ± SEM. *p* values were obtained by data analysis using ANOVA and post hoc analysis to determine significance within groups by Scheffe and Tukey's tests for cells with different cytokine treatments. For statistical analysis on % cell viability and secreted IFN-γ, the values obtained from IL-12 stimulated NK cells were used as baseline for comparison with other cytokine treatments in the presence and absence of TGF-β1. Data from one representative experiment of three independent experiments performed is shown here.

5.2.3 Early Kinetics of TGF-β1 Induced IFN-γ Suppression

Studies by Thomas and Massague using $CD8^+$ T cells showed that TGF-β1 profoundly inhibited expression of cytotoxic proteins, such as Perforin and Granzyme B (*GzmB*), at 24 hours which partially correlated to inhibition of cell proliferation. However, they also demonstrated that the expressions of these proteins were directly and rapidly inhibited within 4 hours by TGF-β1. This indicated that early repression of these proteins was independent and a separate event from TGF-β1 suppressed $CD8^+$ T cell proliferation and growth arrest which occurred at later time points, around 20-24 hours (Thomas & Massague, 2005). Since current data at 6 hours (Figure 5.2, Table 5.1) demonstrated that early IFN-γ production induced by IL-12 was significantly inhibited by TGF-β1 independent of cell proliferation and survival, it was thought to explore further the early kinetics of IFN-γ production. Thus, using early kinetics data in the current study, an attempt was made to minimize the possibility of studying the overlapping inhibitory effects of TGF-β1 on KY-1 cells along with IFN-γ inhibition. For this, KY-1 cells were pre-treated with and without TGF-β1, followed by IL-12 stimulation at multiple time points over 6 hours and the secreted IFN-γ by the cells was assayed by ELISA (Figure 5.3). As seen in this Figure 5.3, the production of IFN-γ was inhibited by about 50% as early as 3 hours. This is an important data, as rapid production of IFN-γ plays a significant role in increasing host resistance to early hours of infections such as *Listeria monocytogenes* (Way & Wilson, 2004; Kang *Et. al.*, 2008), MCMV (Fodil *Et. al.*, 2014). Moreover, significance of such a temporal aspect of cytokine secretion, particularly IFN-γ by NK cells was recently demonstrated in

tumour target cell recognition, consistent with their contribution in immunosurveillence (Fauriat *Et. al.*, 2010). Justifiably, the subsequent mechanistic investigation was based on this early kinetics data.

A) Over 24 hours



B) Over 6 hours

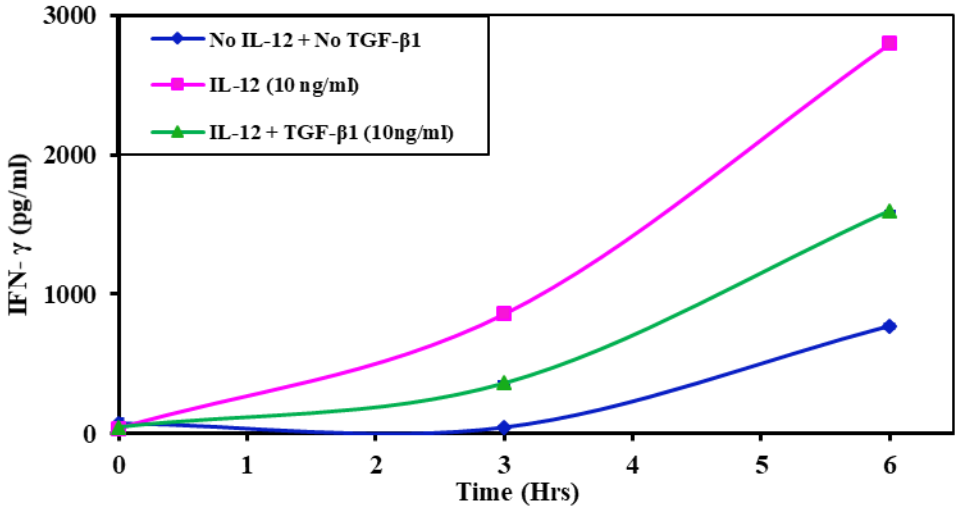


Figure 5.3: Effects of TGF-β1 on IL-12 induced IFN-γ secretion over 24 hours: KY-1 cells (2.5 x 10^5 cells /1.7cm diameter well) were pre-incubated for 30 mins. with or without TGF-β1 (10 ng/ml) followed by addition of IL-12 (10 ng/ml) and cultured for: **A)** over 24 hours with the secreted IFN-γ (ng/ml) assayed at various time points of 0 hour, 3 hours, 6 hours, 16 hours and 24 hours using ELISA. **B)** Detailed graph of inset in (**A)** over the first 6 hours. Results represent the mean of triplicate wells at each point with error bars showing ± SEM, not seen where smaller than symbol size. One representative experiment of three independent experiments performed is shown here.

5.2.4 Effect of TGF-β1 on IL-12 Activated STAT-4 Signalling Pathway in murine NK cells

To understand the molecular basis of TGF-β1-induced suppression of early IFN-γ expression, it was next necessary to investigate the interference of the IL-12 signalling pathway by TGF-β1. Following IL-12 ligation, its receptor associated Janus family kinases (JAK2 and TYK2 kinases) phosphorylates STAT-4 at Tyr-693 (Bacon *Et. al.*, 1995; S. S. Cho *Et. al.*, 1996). However, STAT-4 also requires serine phosphorylation at Ser-721 for complete transcriptional activity and optimal IFN-γ production induced by IL-12 (Morinobu *Et. al.*, 2002). So, the effect of TGF-β1 on IL-12 induced STAT-4 tyrosine and serine phosphorylation was determined.

5.2.4.1 Effect of TGF-β1 on IL-12 Induced Tyrosine Phosphorylation of STAT-4

KY-1 cells were pre-treated with TGF-β1 (10 ng/ml) for 30 mins, followed by IL-12 (10 ng/ml) stimulation for 1 hour, 2 hours, and 3 hours. The culture supernatant was used to measure the secreted IFN-γ using ELISA. From the same culture, cell-lysates were prepared as described in Section 2.7.1. To maximise the detection and identification of phosphorylated STAT-4, cell lysates were treated with pervanadate, protease and phosphatase inhibitors. As seen in Figure 5.4A, tyrosine phosphorylated STAT-4 protein was detected using specific anti-pSTAT-4 Ab (Tyr-693) at 89kDa on a western blot. This blot was then stripped and reprobed with anti-STAT-4 Ab to detect the presence of total STAT-4 protein in the samples (Figure 5.4B). The intensity of these bands (Figure 5.4A and Figure 5.4B) were measured by using software Image J. The results of densitometric analysis are presented in the Table 5.2, as ratios of pSTAT-4/total-STAT-4.

As seen in Figure 5.4A, IL-12 stimulation rapidly induced tyrosine phosphorylation of STAT-4 (Figure 5.4A, lane 2), compared to unstimulated cells (Figure 5.4A, lane 1) at indicated time points, until 3 hours. Further, IL-12-induced tyrosine phosphorylated STAT-4 levels moderately decreased over 3 hours and this decrease was also unaffected in the presence of TGF-β1 (Table 5.2). Importantly, TGF-β1 alone could neither induce any tyrosine phosphorylation of STAT-4 protein (Figure 5.4A, lane 3) nor alter the IL-12 induced tyrosine phosphorylated STAT-4 levels at any of the time

points indicated (Table 5.2 and Figure 5.4A, lane 4). However, a parallel inhibition of IFN-γ at protein levels (about 50%) was observed in the presence of TGF-β1, as shown in Figure 5.4C. This detection of IFN-γ protein secreted served as an internal control in the experimental design.

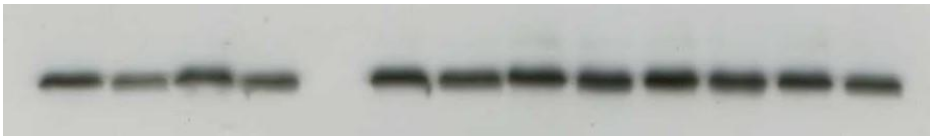
A) Blot: anti-pSTAT-4 (tyrosine)

89kDa

1 hr 2 hr 3 hrs

1 2 3 4 1 2 3 4 1 2 3 4

B) Reprobe: anti-STAT-4



89kDa

1 hr 2 hr 3 hrs

1 2 3 4 1 2 3 4 1 2 3 4

Lanes	1 (Control)	2	3	4
IL-12 (10 ng/ml)	–	+	–	+
TGF-β1 (10ng/ml)	–	–	+	+

C) ELISA for secreted IFN-γ

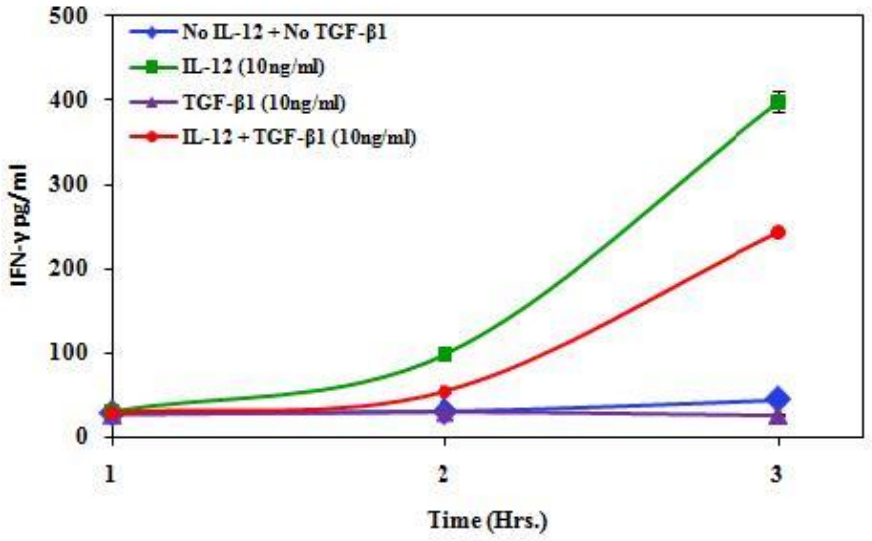


Table 5.2: Densitometric values of pSTAT-4(tyrosine)/STAT-4

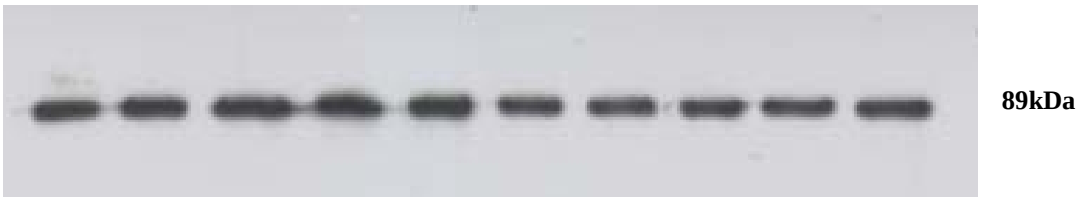
Lanes	1 hr	2hrs.	3 hrs.
1 No IL-12 + No TGF-β1	0.08	0.10	0.08
2 IL-12 (10 ng/ml)	0.43	0.38	0.29
3 TGF-β1 (10ng/ml)	0.12	0.10	0.07
4 IL-12 + TGF-β1(10ng/ml)	0.46	0.34	0.30

Figure 5.4: Effect of TGF-β1 on IL-12 induced tyrosine phosphorylation of STAT-4: KY-1 cells (5 x 10^5 cells/3.4cm diameter well) were pre-treated for 30 mins with or without TGF-β1 (10ng/ml) followed by addition of IL-12 (10 ng/ml) and cultured for 1 h, 2 hours and 3 hours as indicated below each blot. (A) Western blots were immunodeveloped with anti-pSTAT-4 followed by stripping and reprobing the blot with (B) anti-STAT-4. Numbers below each lane in the blot represent cytokine treatment and Table 5.2 summarizes the densitometric values of pSTAT-4 (tyrosine) /STAT-4 ratio. (C) The supernatant was assayed for secreted IFN-γ using ELISA. Above blots are representative of three independent experiments performed.

Additionally, the effect of TGF-β1 at earlier time points of 15 mins and 30 mins on tyrosine phosphorylation of STAT-4 was determined using a similar experimental set-up. The results are shown in Figure 5.5A for pSTAT-4. This blot was stripped and reprobed by anti-STAT-4 Ab for total STAT-4 protein (Figure 5.5B). The densitometric analysis, as shown in Table 5.3, indicated rapid tyrosine phosphorylation of STAT-4 by IL-12 (Figure 5.5A, lane 2) that was not inhibited by TGF-β1 (Figure 5.5A, lane 4) at 15 mins to 2 hours. This result confirms that TGF-β1 does not inhibit IL-12 induced tyrosine phosphorylation of STAT-4, a critical event mediated by activated JAK2 and TYK2 kinases in IL-12 signalling and hence IFN-γ production.

A) Blot: anti-pSTAT-4 (tyrosine)

B) Reprobe: anti-STAT-4



Lanes	1 (Control)	2	3	4
IL-12 (10 ng/ml)	–	+	–	+
TGF-β1 (10ng/ml)	–	–	+	+

Table 5.3: Densitometric values of pSTAT-4(tyrosine)/STAT-4 at 15 mins. until 2 hrs.

Lanes	15 mins	30 mins	1 hr	2hrs.
1 No IL-12 + No TGF-β1	0.18	-	-	-
2 IL-12 (10 ng/ml)	0.54	0.53	0.54	0.50
3 TGF-β1 (10ng/ml)	0.17	-	-	-
4 IL-12 + TGF-β1(10ng/ml)	0.52	0.52	0.55	0.51

Figure 5.5: Effect of TGF-β1 on IL-12 induced tyrosine phosphorylation of STAT-4 at 15 mins until 2 hrs: KY-1 cells (5 x 10^5 cells/3.4cm diameter well) were pre-treated for 30 mins. with or without TGF-β1 (10ng/ml) followed by addition of IL-12 (10 ng/ml) for 15 mins, 30 mins, 1 h and 2 hours as indicated below each blot. (A) Western blots were immunodeveloped with anti-pSTAT-4 followed by stripping and reprobing the blot with (B) anti-STAT-4. Numbers below each lane in the blot represents cytokine treatment and Table 5.3 shown summarises the densitometric values of pSTAT-4/STAT-4 ratio (tyrosine). Above blots are representative of three independent experiments performed.

5.2.4.2 Effect of TGF-β1 on IL-12 Induced Serine Phosphorylation of STAT-4

In addition to Tyr693 phosphorylation on IL-12 stimulation, STAT-4 also undergoes phosphorylation at Ser721 (S. S. Cho *Et. al.*, 1996; Visconti *Et. al.*, 2000). Since the results from previous experiment showed that TGF-β1 did not inhibit tyrosine phosphorylation of STAT-4 (Figure 5.4 and Figure 5.5), it might possibly inhibit IL-12-induced serine phosphorylation of STAT-4. Therefore, the blot used for detection of tyrosine STAT-4, as shown in the previous experiment (Figure 5.4A) was stripped and then reprobed using specific anti-pSTAT-4 Ab that detected phosphorylated STAT-4 at serine 721 (as seen in Figure 5.6). This immunoblot (Figure 5.6) was then further stripped and reprobed to detect the levels of total STAT-4 protein in the samples (Figure 5.4B). It is also important to note that each step of stripping was carefully performed to minimise loss of protein from the blot. Prior to reprobing, the efficiency of stripping method was checked to ensure removal of primary Ab as described in Section 2.9.5. The blots were analysed using Image J and the results of this densitometric analysis are shown in Table 5.4. The level of phosphorylation was determined by the ratio of p-STAT-4 (serine) to total STAT-4 protein.

As seen in the Figure 5.6, serine phosphorylated STAT-4 was detected in unstimulated cells indicating base-line expression levels (Figure 5.6, lane 1), that was not significantly induced on IL-12 stimulation (Figure 5.6, lane 2). Further, TGF-β1 treatment alone (Figure 5.6, lane 3) and in the presence of IL-12 (Figure 5.6, lane 4) was unable to affect the serine phosphorylated STAT-4 levels at any of the time points indicated. Thus, the results showed that even though TGF-β1 inhibited IFN-γ secretion (Figure 5.4C), it neither inhibited tyrosine phosphorylation nor altered the constitutive levels of serine phosphorylated STAT-4 in murine NK cells. Hence, the data suggested that the underlying mechanism in the suppression of IL-12-stimulated IFN-γ secretion

by TGF-β1 was independent of interference with activation of key component of IL-12 signalling, STAT-4.

Reprobe: anti-p-STAT-4 (serine)

89kDa

1 hr 2 hr 3 hrs

1 2 3 4 1 2 3 4 1 2 3 4

Lanes	1 (Control)	2	3	4
IL-12 (10 ng/ml)	–	+	–	+
TGF-β1 (10ng/ml)	–	–	+	+

Table 5.4: Densitometric values of pSTAT-4 (serine) /STAT-4

Lanes	1 hr	2hrs.	3 hrs.
1 No IL-12 + No TGF-β1	0.48	0.48	0.42
2 IL-12 (10 ng/ml)	0.55	0.49	0.48
3 TGF-β1 (10 ng/ml)	0.52	0.45	0.49
4 IL-12 + TGF-β1(10 ng/ml)	0.55	0.46	0.51

Figure 5.6: Effect of TGF-β1 on IL-12 induced serine phosphorylation of STAT-4: Tyrosine phosphorylated STAT-4 blot (Fig 5.4A) was stripped and reprobed with anti-pSTAT-4 (serine) Fig 5.6. This was finally stripped and reprobed with anti-STAT-4 as shown in (Fig.5.4B). Numbers below each lane in the blot represents cytokine treatment and Table 5.4 shown summarises the densitometric values of pSTAT-4/STAT-4 ratio (serine). Above blot is representative of three independent experiments performed.

5.2.5 Effect of TGF-β1 on T-bet Expression in IL-12 Stimulated murine NK Cells

As mentioned earlier in Section 1.8.2.1 that besides STAT-4, another factor upregulated by IL-12 and positively implicated in IFN-γ expression in both T and NK cells is T-bet (Szabo *Et. al.*, 2000; Szabo *Et. al.*, 2002; Sullivan *Et. al.*, 2003). Moreover, TGF-β1 controlled IFN-γ expression in T-cells was shown to be mediated by downregulation of T-bet (Neurath *Et. al.*, 2002; Gorelik *Et. al.*, 2002a; J. T. Lin *Et. al.*, 2005). Therefore, it was important to examine next the effect of TGF-β1 on T-bet expression in KY-1 cells. The cell lysate prepared and used for STAT-4 phosphorylation studies (in earlier Section 5.2.4) was also used in this experiment for the detection of the T-bet protein. This was because the experimental design and conditions for both the studies were the same. Consistent with the previous study (Szabo *Et. al.*, 2000), it was seen in Figure 5.7A that a band for T-bet was detected at approximately 62 kDa, using a specific monoclonal antibody, anti-T-bet, on the western blot. The blot was stripped and reprobed for β-actin that was used as a loading control (Figure 5.7B). Blots were then analyzed using Image J. Table 5.5 summarizes the densitometric analysis of T-bet detected against β-actin levels on the same blot. The data revealed that T-bet expression was neither induced by IL-12 (Figure 5.7A, lane 2) nor affected by TGF-β1 (Figure 5.7A, lane 4).

T-bet expression observed in unstimulated cells was not augmented by IL-12 stimulation (Figure 5.7A, lanes 1 & 2). This suggests a constitutive or a base-line expression of T-bet in murine NK cells. Similar constitutive T-bet expression was observed in another NK cell line, YT but was not detected in T cell lines (EL-4 or Jurkat) (Szabo *Et. al.*, 2000). This suggests that such constitutive T-bet expression might have a cell-specific function in NK cells than in T cells. Nevertheless, the base-line expression of T-bet was not affected by TGF-β1 treatment (Figure 5.7A, lanes 3 & 4), even though, in parallel, inhibition of IFN-γ protein levels was detected by ELISA (Figure 5.4C). The results indicate that T-bet does not drive the early production of IFN-γ by IL-12 (3 hours). Thus, TGF-β1 induced early suppression of NK cell IFN-γ is independent of direct role of T-bet.

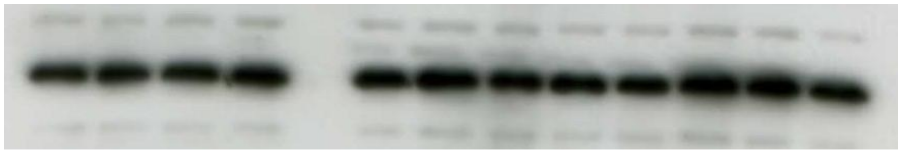
A) Blot: anti-T-bet

62kDa

1 hr 2 hr 3 hrs

1 2 3 4 1 2 3 4 1 2 3 4

B) Reprobe: anti-β-Actin



48kDa

1 hr 2 hr 3 hrs

1 2 3 4 1 2 3 4 1 2 3 4

Lanes	1 (Control)	2	3	4
IL-12 (10 ng/ml)	–	+	–	+
TGF-β1 (10ng/ml)	–	–	+	+

Table- 5.5: Densitometric values of T-bet/β-actin

Lanes	1 hr	2hrs.	3 hrs.
1 No IL-12 + No TGF-β1	1.04	1.14	1.11
2 IL-12 (10ng/ml)	1.06	1.03	0.99
3 TGF-β1 (10ng/ml)	1.14	1.05	0.99
4 IL-12 + TGF-β1 (10ng/ml)	1.09	1.09	1.01

Figure 5.7: Effect of TGF-β1 on T-bet expression in murine NK cells: KY-1 cells (5 x 10^5 cells/3.4 cm diameter well) were pre-treated for 30 mins with or without TGF-β1 (10 ng/ml) followed by stimulation with IL-12 (10 ng/ml) for 1 h, 2 hours and 3 hours as indicated below each blot. (**A**) Western blots were immunodeveloped with anti-T-bet followed by stripping and reprobing the blot with (**B**) anti-β-actin. Numbers below each lane in the blot represents cytokine treatment and Table 5.5 shown summarises the densitometric values of T-bet/β-actin ratio. Background noise for both the blots (A) and (B) were not adjusted in densitometric analysis and resulted in T-bet/β-actin ratio values around '1'. The cell culture supernatant was assayed for secreted IFN-γ using ELISA (Figure 5.4C). Above blots are representative of three independent experiments performed.

5.2.6 Effect of TGF-β1 on IFN-γ Gene Expression

The data until now has demonstrated that the signalling crosstalk between TGF-β1 and IL-12 is independent of direct involvement of key transcriptional factors, STAT-4 (Figure 5.4, 5.6) and T-bet (Figure 5.7) regulating IFN-γ gene expression. However, the effective production of IFN-γ requires binding of various transcription factors onto the IFN-γ promoter and its intronic region where they act as either activators or repressors, controlling IFN-γ gene transcription (Penix *Et. al.,* 1993; Sweetser *Et. al.*, 1998; Tato *Et. al.*, 2004; Zhou *Et. al.*, 2004). Several studies observed reduced IFN-γ protein levels in the presence of TGF-β1 and reported a parallel reduction in IFN-γ transcripts, which suggests the possibility of transcriptional regulation of IFN-γ gene by TGF-β1 (Sudarshan *Et. al.*, 1999; Hayashi *Et. al.*, 2003; Laouar *Et. al.*, 2005; J. T. Lin *Et. al.*, 2005; Thomas & Massague, 2005). Nonetheless, the molecular basis to this transcriptional regulation is still unclear.

Interestingly, a study in TCR-activated primary $CD8^+$ T cells demonstrated that TGF-β1-induced transcription factors, CREB and ATF-1 acted as Smad-binding partners in the promoter repression of a cytotoxic gene, granzyme B, *GzmB* (Thomas & Massague, 2005). Additionally, in the suppression of IFN-γ promoter activity, direct binding of ATF-1 and Smads 2/3 to the IFN-γ promoter region that spanned across -366 to -16 bp from the TSS (Transcription Start Site) was observed through ChIP (Chromatin Immunoprecipitation) analysis. Therefore, based on the presence of ATF-1 and Smad-binding sites close to each other within the IFN-γ promoter region (-300/+4bp), they proposed a mechanism in IFN-γ inhibition to be similar to the repression of *GzmB* promoter activity by TGF-β1. Therefore, one possible mechanism by which TGF-β1 inhibits might be direct interference with the transcriptional capability of the IFN-γ promoter. One mode of studying transcriptional repression is analysing promoter function using reporter gene assays. Taken together, this prompted interest to investigate the effect of TGF-β1 on IFN-γ promoter activity using luciferase reporter assays. This should enable identification of a *cis*-acting regulatory region/elements within the IFN-γ promoter that is susceptible to TGF-β1 signalling in NK cells.

In order to conduct this part of the experimental study, it was important to decide on the appropriate size or region of the IFN-γ promoter that should be considered in the

reporter vector for transient expression studies. Most studies used different IFN-γ promoter construct sizes ranging from <100bp to 3kb upstream of the TSS of the IFN-γ gene in reporter assays. These reporter constructs were successfully expressed and exhibited inducible promoter activity in both T and NK cell lines (H. Zhu *Et. al.*, 2001; Soutto *Et. al.*, 2002; J. Y. Cho *Et. al.*, 2003; Bream *Et. al.*, 2003). Essentially, the 'core' IFN-γ promoter region was identified as -108bp length upstream of a 5' human IFN-γ gene that reflected expression of the endogenous IFN-γ gene and contained proximal (-73 bp to -48 bp) and distal (-96 bp to -80 bp) *cis*-regulatory elements. These elements are conserved across species and required for basal promoter function (Penix *Et. al.*, 1993; Aune *Et. al.*, 1997; Soutto *Et. al.*, 2002). Further, 5' upstream regions of the IFN-γ gene such as -280 to -180 bp (Ye *Et. al.*, 1994) and -445 to -415 bp (Soutto *Et. al.*, 2002) had been previously described to contain binding sites for various transcription factors including STATs, NFAT, NF-kB, AP-1, ATF-CREB, YY-1, T-box, GATA. These proximal promoter regions were shown to contribute to the regulation of IFN-γ gene transcription in response to TCR and/or cytokine stimulations (Young, 1996; Sica *Et. al.*, 1997; Sweetser *Et. al.*, 1998; K. M. Murphy *Et. al.*, 2000; Nakahira *Et. al.*, 2002). For most of these factors, the region on the IFN-γ promoter to which it binds, has been determined either *in vitro* or *in vivo*.

Since, the current study has been focused on two important transcription factors in IFN-γ expression, STAT-4 and T-bet, it was necessary to understand their role in *trans*-activation of the IFN-γ gene. Importantly, STAT-4 has been shown to *trans*-activate IFN-γ gene by either direct binding to the STAT-binding element (-236 bp) on the IFN-γ promoter (X. Xu *Et. al.*, 1996) or to enhance binding of other factors like NF-kB (D. Robinson *Et. al.*, 1997) or AP-1 (-190 bp) in IFN-γ gene expression (Barbulescu *Et. al.*, 1998; Nakahira *Et. al.*, 2002). This was evident from STAT-4 $^{-/-}$ T cells which produced AP-1 levels similar to wild-type but failed to exhibit AP-1 binding in the presence of IL-12 (Park *Et. al.*, 2005). Similarly, functional T-bet responsive elements have been determined on the proximal region of the IFN-γ promoter (Soutto *Et. al.*, 2002; J. Y. Cho *Et. al.*, 2003), as a mutation in this region resulted in significant loss of T-bet augmented IFN-γ reporter activity in T cells (J. Y. Cho *Et. al.*, 2003; Tong *Et. al.*, 2005). Since, the current results demonstrated constitutive expression of T-bet that is not directly involved in IFN-γ induction (Figure 5.7), this protein might be indirectly involved in induction of endogenous IFN-γ promoter activity. Previous studies have suggested an indirect role of T-bet, through co-operation with other transcription

factors in IFN-γ promoter activation (Soutto *Et. al.*, 2002). A T-bet responsive unit (TRU) was identified within -565 bp to -415 bp region of the IFN-γ promoter (Soutto *Et. al.*, 2002). However, Cho *Et. al.* (2003) further demonstrated that T-bet functionally mediates IFN-γ promoter activity through multiple T-box 'half-sites', also called monomeric Brachyury sites located within -300bp of IFN-γ promoter. One of the most important of T-bet 'half-sites' was located between -66 bp and -57 bp region of very proximal IFN-γ promoter. This 9bp sequence was shown to be required, even in the presence of an upstream TRU element (J. Y. Cho *Et. al.*, 2003). Interestingly, both these T-bet interaction sites (-565/-415 bp and -66/-57 bp) on the IFN-γ promoter have been located close to consensus core binding sequences for several other transcription factors, mentioned above (H. Zhu *Et. al.*, 2001; Soutto *Et. al.*, 2002; J. Y. Cho *Et. al.*, 2003; Tong *Et. al.*, 2005). This suggested that co-operation from other factors might come into play to enhance T-bet function in IFN-γ promoter activation.

Collectively, this specific information was considered in the selection of an appropriate size or a region of the IFN-γ promoter for the subsequent transient reporter assays. Therefore, a reporter vector (denoted as pFL-IFN) containing an insert of -777bp IFN-γ promoter region upstream of a TSS of the IFN-γ gene was selected. This pFL-IFN vector was constructed using a firefly luciferase plasmid, pBS-Luc (as detailed in Section 2.10). The pFL-IFN was one of the reporter plasmids received as a kind gift from Dr. Howard A. Young (National Cancer Institute, National Institutes of Health, Frederick, MD). A previous study by Zhu *Et. al.* (2001) has reported the use of this particular plasmid along with other IFN-γ promoter constructs of varying sizes, ranging from -59 to -3489 bp upstream of the TSS in the transient transfection of EL-4 cells (T-lymphoma cells). These promoter constructs exhibited inducible expression on P/I stimulation, with only slight differences in the levels of inducibility between them. This indicated that the chosen pFL-IFN reporter vector in the current study was inducible, when transiently expressed in a cell line, however the strength of induction may vary with the stimuli used. In the current study, the expression of this pFL-IFN reporter construct on IL-12 stimulation was examined in transiently transfected NK cells. Also, the effect of TGF-β1 on IL-12-induced promoter activity was determined. In case IL-12 alone stimulation did not result in significant induction of IFN-γ reporter activity, a positive control was included which contained IL-12 plus IL-18 co-stimulation. IL-18 alone is a weak inducer of IFN-γ expression, however, it is synergistic in action with IL-12 and had been shown to potently activate IFN-γ reporter activity (Barbulescu *Et.*

al., 1998; J. Yang *Et. al.*, 1999; Nakahira *Et. al.*, 2002). Importantly, this control indicates that the IFN-γ promoter fragment cloned into the luciferase reporter vector was active to specific cytokine stimulations used.

Additionally, at this point, a bioinformatic application was briefly used as a screening tool to further facilitate the identification of regulatory elements that might be significant in TGF-β1 regulated repression of IL-12-induced IFN-γ expression. The selected region of the IFN-γ promoter (-777bp to +48bp) that is used in the transfection experiments, was analysed for the potential transcription factor binding sites using the program, MatInspector 8.0.5. This Genomatrix software suite V2.5 based program identifies a series of 4bp core sequences for each possible transcription factor binding within the sequences of query promoter. The outcome of the search was a broader set of transcription factors in addition to those mentioned above with their consensus binding sequence-motif. Some of the important regulatory regions residing within the selected size of IFN-γ promoter have been highlighted in Figure 5.8. In the event that TGF-β1 repressed IFN-γ promoter activity is observed, the results of this bioinformatic analysis will potentially be used in mapping the region/element on the promoter which correlates to binding factors.

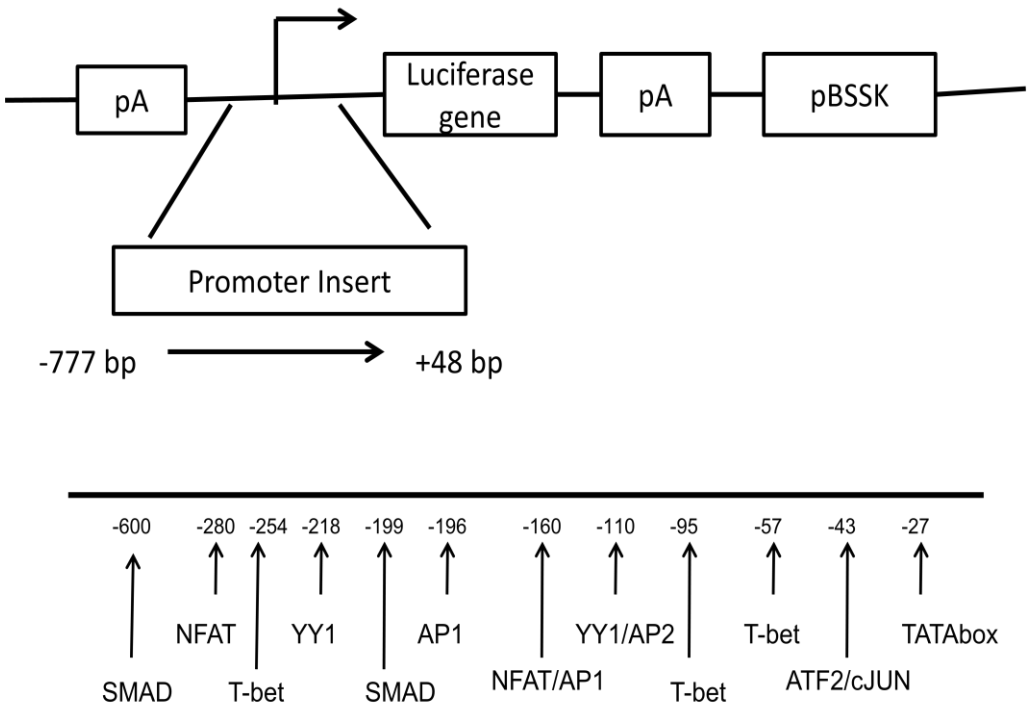


Figure 5.8: Schematic representation of IFN-γ promoter reporter vector and putative transcription factor binding sites within the -777/+48 promoter element: IFN-γ promoter region of -777bp to +48bp used was inserted upstream of luciferase gene into pBS-Luc vector to generate the pFL-IFN reporter vector. This pFL-IFN vector was transiently transfected into KY-1 cells as described in the Materials and Methods. **M**embers of various regulatory transcription factor families known to bind within the selected IFN-γ promoter region (Penix *Et. al.*, 1993; Sweetser *Et. al.*, 1998; Tato *Et. al.*, 2004; Zhou *Et. al.*, 2004) and identified by the programme MatInspector 8.0.5 are shown.

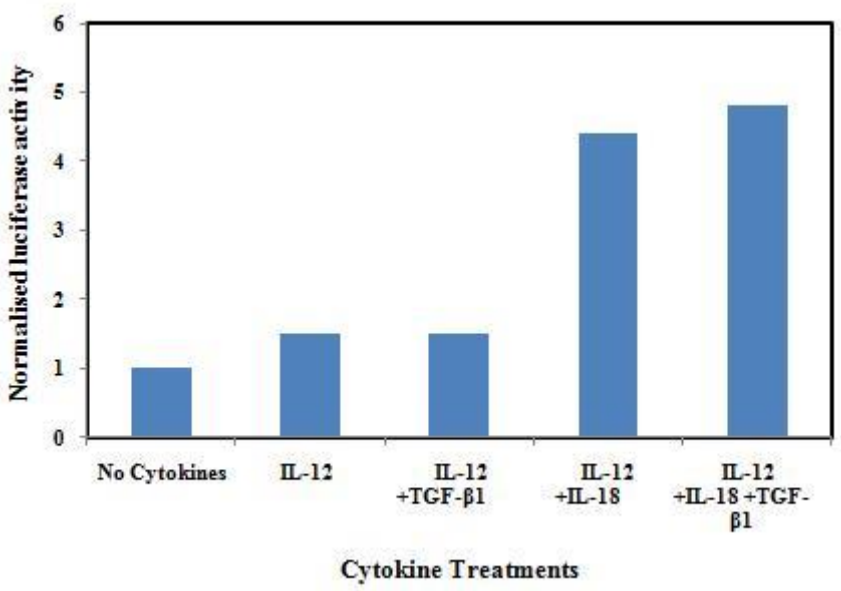
Moreover, prior to performing this experimental study, it was also required to understand the transfection efficiency of the NK cell line (KY-1) used. In general, NK cells are known to be the difficult cell lines to transfect, therefore, many laboratories focus their investigation on regulation of IFN-γ expression using T cells (Biron *Et. al.*, 1999; Bream *Et. al.*, 2004). To our knowledge, this was the first study that attempted transient transfection of this NK cell line, KY-1, using the Amaxa nucleofection method (described in section 2.11.1). Accordingly, KY-1 cells were transiently co-transfected with the murine IFN-γ reporter vector, pFL-IFN, and pRL-TK vectors. pRL-TK plasmid (*Renilla* luciferase reporter gene) is a commercial plasmid, used as an internal control to normalise transfection efficiency. The details of these reporter vectors are in Section 2.10.

Transiently co-transfected cells were left unstimulated (No cytokines), treated with or without TGF-β1 for 30 mins. Subsequently, these cells were stimulated with either IL-12 alone or with a combination of IL-12 plus IL-18 for 3 hours in the presence or absence of TGF-β1, as seen in Figure 5.9. After stimulation with cytokines, cells were lysed to detect the reporter activity using Dual-Luciferase® Reporter Assays, as described in Section 2.11.2. The transiently expressed IFN-γ reporter activity was measured as an increase in firefly luciferase activity (experimental) normalised to *Renilla* luciferase activity (internal control). After normalisation for transfection efficiency (as shown in Figure 5.9A), IL-12 stimulation resulted in ~50% increase in the level of IFN-γ promoter activity (bar-2) in comparison to the 'basal' promoter activity from unstimulated cells (bar-1). Importantly, this IL-12-induced reporter activity was unaffected by the treatment with TGF-β1 (bar-3). As expected and consistent with other studies (Barbulescu *Et. al.*, 1998; Nakahira *Et. al.*, 2002), IL-12 plus IL-18 co-stimulation resulted in relatively, much higher expression of IFN-γ reporter activity (~4 fold increase) (bar-4). As expected, it served as a positive control in observing the activation of IFN-γ promoter fragment. Still, TGF-β1 was unable to inhibit this synergistic induction of transiently expressed IFN-γ promoter construct (bar-5). Thus, the results clearly demonstrated that the presence of TGF-β1 failed to suppress the IFN-γ promoter-driven luciferase activity induced by IL-12 alone or with IL-12 plus IL-18.

In addition to measuring the reporter activity from cells, the culture supernatant was used to assay for endogenous IFN-γ production using ELISA (Figure 5.9B). This assay served as an internal control that reflected the intact activities of exogenously added cytokines for stimulation. It also acted as an internal control to the response of the endogenous or native IFN-γ promoter under the experimental conditions used. As seen in Figure 5.9B, expectedly, IL-12 stimulation resulted in IFN-γ production from transiently transfected KY-1 cells (bar-2) and the presence of IL-18 with IL-12 strongly augmented this production (bar-4). Also, TGF-β1 significantly inhibited ~50% IFN-γ production induced by IL-12 alone (bar-3) or with combination of IL-12 plus IL-18 (bar-5). This inhibitory effect of TGF-β1 on transfected KY-1 cells was found comparable to the effect observed with non-transfected KY-1 cells in the previous experiments (Figure 5.1).

Nonetheless, the expression level of the exogenous IFN-γ promoter construct induced by IL-12 alone stimulation (Figure 5.9A) was not comparable to the native IFN-γ promoter activity in these transient transfections (Figure 5.9B). This shows that the transcriptional activity of the IFN-γ promoter construct used here does not reflect the transcription of the native IFN-γ gene and indicates the requirement for analysing larger promoter constructs in future experiments. This extended region might provide additional binding sites or elements in the IFN-γ promoter for the transcription factors induced by IL-12 and hence induce stronger transcriptional activation of the promoter construct. Such a study might lead to a complete 'switch-on' of IFN-γ gene expression, to investigate the effect of TGF-β1. Moreover, the analysis of the proximal IFN-γ promoter region within -777bp to +48bp that appeared to be important for various transcription factors (Figure 5.8) was not responsive to the presence of TGF-β1. This suggests that the repression of the native IFN-γ gene by TGF-β1 may require distal regulatory elements at >777bp upstream of the TSS. Thus, as suggested earlier, to advance the experimental work, it would be interesting to increase the size of the IFN-γ promoter inserts into the luciferase vector and investigate its inhibition by TGF-β1. Once a defined region of the IFN-γ promoter that is responsive to TGF-β1 is determined, targeted deletion or mutational analysis of functional regulatory elements based on bioinformatics data can be addressed in the regulation of IFN-γ gene by TGF-β1.

(5.9A)



(5.9B)

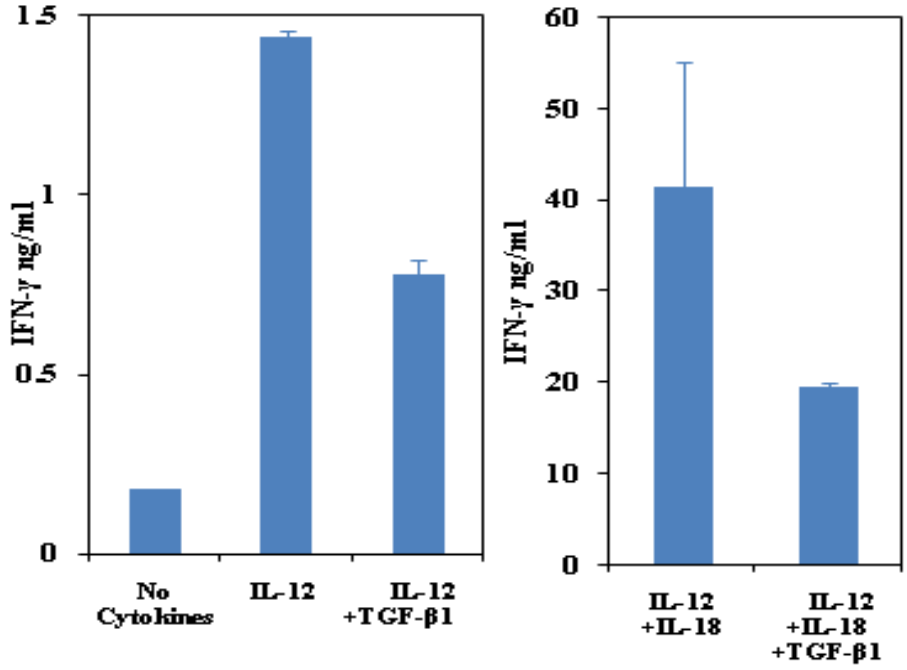


Figure 5.9: Effect of TGF-β1 on exogenous and endogenous IFN-γ promoter activity: KY-1 cells were transiently co-transfected with pFL-IFN and pRL-TK plasmids. Transfected cells were treated with or without TGF-β1 (10 ng/ml) for 30 mins, subsequently cells were stimulated by either IL-12 (10 ng/ml) alone or IL-12 plus IL-18 (100 ng/ml) for 3 hrs. (A) Cells were lysed to detect luciferase reporter activity. Results shown are expressed as ratio of relative light units (RLU) of firefly (F) to renilla (R) that are normalised to the fold change in activity by referring the ratio obtained in untreated (No cytokines) transfected wells as '1'. (B) The supernatant culture media from transfected cells was collected and assayed by ELISA to measure endogenous IFN-γ protein levels. Error bars show ± SD. The data are representative of three independent experiments each with two transfected wells per experiment.

5.3 Discussion

Prior to the current investigation, several published works reported inhibition of IFN-γ production by TGF-β1. However, the signalling crosstalk between TGF-β1 and IL-12 in IFN-γ production was not well understood. Moreover, as described earlier in chapter 1 (Section 1.8.2), conflicting reports over the inhibitory effects of TGF-β1 on IL-12 receptor-proximal events highlighted the need for further investigation. This chapter, therefore, sought to investigate the underlying mechanism in the regulation of IL-12-induced IFN-γ production by TGF-β1 in NK cells and hence, enhances understanding towards the pivotal role of NK cell-derived IFN-γ in early innate immune responses to tumours and opportunistic infections such as in immunocompromised individuals inAIDS. Thus, this study has potential implications for NK cell functions in health and disease.

Particularly, an attempt has been made to decipher the interference of TGF-β1 on IL-12 signalling elements in IFN-γ production and gene expression. Interestingly, the current results demonstrated that early IFN-γ production (3-6 hours) was suppressed by TGF-β1 (Figure 5.3), which indicated a mechanism independent of cell proliferation. The inhibitory effect of TGF-β1 on IFN-γ production observed in the current study (Figure 5.3) is in line with a number of previous studies (Bellone *Et. al.*, 1995; Bright & Sriram, 1998; Sudarshan *Et. al.*, 1999; Pardoux *Et. al.*, 1999; Thomas & Massague, 2005; Yu *Et. al.*, 2006; Lindgren *Et. al.*, 2011; Marcoe *Et. al.*, 2012; Lewis *Et. al.*, 2015). However, these previous studies demonstrated the effect over longer hours of treatment with TGF-β1 (overnight to 48 hours) which, in part, might include contributions from other suppressive actions of TGF-β1 such as anti-proliferation (Bellone *Et. al.*, 1995; Bright & Sriram, 1998; Wilson *Et. al.*, 2011) or induction of factors in cell-cycle inhibition (Ahuja *Et. al.*, 1993; Bellone *Et. al.*, 1995). Indeed, the current results highlight the independence of two TGF-β1 regulated pathways, namely, cell proliferation and IFN-γ production. The data also indicates (Figure 5.2 and Table 5.1) that reduced IFN-γ secretion was not due to reduction in numbers of NK cell population in the presence of TGF-β1. This data is critical in immune responses *in vivo* that are dependent on NK cells, and prior to activation of T cells. Such a scenario could arise during early hours of infections with intracellular bacterial or viral pathogens (French & Yokoyama, 2003). Studies with murine infectious models of LM (*Listeria monocytogenes*) (Way & Wilson, 2004; S. J. Kang

Et. al., 2008) and CMV (Cytomegalovirus) (Fodil *Et. al.*, 2014; Lewis *Et. al.*, 2015) have shown that IFN-γ production by NK cells and not by T cells is associated with providing resistance to infections.

To investigate the potential interference of TGF-β1 in IFN-γ production, the IL-12-activated JAK-STAT pathway in NK cells was examined. IL-12 signals through the IL-12R-associated Janus family kinases, JAK2 and TYK2, which in turn tyrosine phosphorylates and activates STAT-4 (Bacon *Et. al.*, 1995; O'Shea, 1997). In the current study, IL-12 induced tyrosine phosphorylation of STAT-4 was not affected by TGF-β1, despite inhibition of IFN-γ secretion (Figure 5.4, 5.5). This suggested that IL-12 receptor-proximal events such as activation of JAKs and kinase activity was normal in the presence of TGF-β1. These results are also in agreement with the previously reported findings of Sudarshan *Et. al.* (1999) who demonstrated using T and NK cells that pre-treatment with TGF-β1 did not inhibit IL-12-stimulated tyrosine-phosphorylation of STAT-4 and its DNA binding activity. Moreover, the current study extends their findings by demonstrating that the inhibitory effect of TGF-β1 was also independent of serine phosphorylation of STAT-4.

Although, tyrosine phosphorylation is the primary regulator of STAT activity, maximal activation of STAT requires both tyrosine and serine phosphorylation (S. S. Cho *Et. al.*, 1996; Visconti *Et. al.*, 2000). Phosphorylation of STAT-4 at Ser721 is required for IL-12 induced full transcriptional activity and IFN-γ production (Morinobu *Et. al.*, 2002). In the current study, IL-12 stimulation did not result in any increased levels of serine phosphorylation of STAT-4 (Figure 5.6). Rather, STAT-4 was detected to be constitutively serine-phosphorylated in this NK cell line, KY-1. One possible explanation for the constitutive levels of serine phosphorylated STAT-4 could be maintenance of these KY-1 cells in IL-2, which is one of the known stimulants of p38 MAPK (Hunt *Et. al.*, 1999). The p38 MAPK pathway mediates serine phosphorylation of STAT-4, with the effect that the inhibitors of p38 MAPK inhibit IFN-γ production (Visconti *Et. al.*, 2000).

Alternatively, the KY-1 cell line used in the current study was obtained from a p53 knockout mouse which may contain constitutively activated p38 from MAPK family. This is because, deletion of p53 gene (a tumour-suppressor gene) gives these NK cells immortality similar to a tumour cell line. A correlation between constitutive expression

of serine phosphorylated STAT-4 to the constitutively activated p38 MAPK kinase in human T-cell leukaemia virus type I (HTLV-1) transformed T cell lines has been reported (Higashi *Et. al.*, 2005). Similar to STAT-4, other STATs, like STAT-1 and STAT-3, were also reported to be constitutively serine phosphorylated in leukaemic T cells from chronic lymphocytic leukaemia patients (Frank *Et. al.*, 1997). Thus, there could be a possible contribution of the p38 MAPK pathway in constitutive serine phosphorylation of STAT-4 in KY-1 cells. Importantly, TGF-β1 treatment did not result in the reduction or inhibition of this constitutive level of serine phosphorylated STAT-4. This suggests that even if STAT-4 was serine phosphorylated by constitutive p38 MAPK activity, it is quite unlikely for TGF-β1 to have a profound effect on this signalling pathway to reflect in IFN-γ production. Consistent with this, Trotta *Et. al.* (2008) also showed that TGF-β1 did not inhibit early signalling intermediates such as p38 kinase, STAT-4/5 in IFN-γ production by NK cells via CD-16 and IL-12 activation. Overall, the data strongly supports a STAT-4 independent role of TGF-β1 in suppression of IL-12-induced IFN-γ production (Section 5.2.4).

IFN-γ has been identified as one of the target genes for a T-box transcription factor, T-bet (Szabo *Et. al.*, 2002; Townsend *Et. al.*, 2004). In the current study, T-bet expression was detected at similar levels in IL-12 stimulated and unstimulated cells in KY-1 cells. This means that T-bet is constitutively expressed in these NK cells and does not correlate to rapid IFN-γ production observed on short-term (3 hours) stimulation with IL-12. A similar early burst of IFN-γ release by NK cells was shown to be unaffected by the absence of T-bet on stimulation with IL-12/IL-18 *in-vitro* (Townsend *Et. al.*, 2004). Moreover, it has been shown that T-bet dependent IFN-γ production varies with the type of stimulation used. T-bet was shown to strongly increase IFN-γ production in T-bet expressing Th2 cells or STAT-4$^{-/-}$ T cells when stimulated with P/I but did not strongly influence IFN-γ production when stimulated with antigen/APC or IL-12 + IL-18 (Afkarian *Et. al.*, 2002). Further, cytokine-induced IFN-γ expression remained dependent on STAT-4 even in the presence of ectopically expressed T-bet in T cells (Afkarian *Et. al.*, 2002). Consistent with this, the current findings also showed that in the presence of T-bet, STAT-4 signalling in response to IL-12 could induce IFN-γ. In this context, recent studies have shown that neither retroviral T-bet expression in STAT-4$^{-/-}$T-bet$^{-/-}$ double deficient T cells nor IL-27 induced Tbx21 expression in STAT-4-deficient T cells was sufficient to increase IFN-γ production compared to WT. However, ectopic T-bet expression in T-bet deficient cells fully induced IFN-γ

expression to WT levels (Thieu *Et. al.*, 2008). This indicated that while STAT-4 is critical, T-bet without STAT-4 does not induce IFN-γ production (Thieu *Et. al.*, 2008; J. Zhu *Et. al.*, 2012). Further these studies have shown that both T-bet and STAT-4 act in collaboration to induce IFN-γ production (J. Zhu *Et. al.*, 2012) by mediating distinct chromatin modifications such as histone acetylation at the *Ifng* locus (Thieu *Et. al.*, 2008; Balasubramani *Et. al.*, 2010; Balasubramani *Et. al.*, 2014). Therefore, the constitutively expressed T-bet might possibly enable the recruitment of activated STAT-4 or other factors in IL-12 stimulated IFN-γ production (Thieu *Et. al.*, 2008; Kurktschiev *Et. al.*, 2014).

More importantly, the constitutive expression of T-bet observed in the current study was found unaltered in the presence of TGF-β1 (Figure 5.7). In line with this, another study has shown that T-bet expression was not affected by TGF-β-mediated IFN-γ suppression in NK cells from healthy individuals that was also consistent with T-bet expression in cancer patient-derived NK cells (Lindgren *Et. al.*, 2011). The molecular mechanism by which T-bet positively regulates IFN-γ gene expression has been well addressed using T cells, however it remains insufficiently explored in NK cells. As a classic transcription factor, T-bet directly binds to the sequences on the IFN-γ promoter and induces transcription (Szabo *Et. al.*, 2000). Indirectly, in collaboration with other transcriptional factors, T-bet has been shown in T cells to influence the induction of IFN-γ transcription or block the recruitment of transcriptional repressors that regulate IFN-γ gene expression (Soutto *Et. al.*, 2002; J. Y. Cho *Et. al.*, 2003; Djuretic *Et. al.*, 2007). Current data indicates that TGF-β1 suppressed early IFN-γ production is not through a mechanism that involves direct transcriptional function of T-bet. Supporting this finding, the activity of -777 to +48bp IFN-γ promoter construct that contained multiple functional sites for T-bet was investigated in the presence of TGF-β1. Results from the reporter assays demonstrated that induction of IFN-γ promoter activity on IL-12 stimulation remained unresponsive to TGF-β1 (Figure 5.9a), inspite of the presence of T-bet responsive cis-regulatory elements within 300bp upstream of TSS of IFN-γ gene (J. Y. Cho *Et. al.*, 2003). Some of these sites have been highlighted in Figure 5.10 (Green region). Thus, it can be concluded that the potential T-bet interaction sites in the proximal region of IFN-γ promoter were not the target regulatory elements in TGF-β1-induced early IFN-γ repression. However, this does not rule out the possibility that T-bet in co-operation with other transcription factors might function in *trans*-activation of IFN-γ promoter.

Notably, the IFN-γ promoter construct used in the reporter assays also contained the binding sites for various other factors important in IFN-γ expression such as AP-1, YY-1, NF-AT, NF-kB, and STATs, in addition to T bet (Ye *Et. al.*, 1994; K. M. Murphy *Et. al.*, 2000; Soutto *Et. al.*, 2002). Still, TGF-β1 was unable to repress IL-12-induced IFN-γ promoter activation (Figure 5.9a). This, to some extent, suggests that TGF-β1 does not target the indirect role of T-bet in influencing IFN-γ promoter activity. However, it will be useful in future to confirm the role of this constitutively expressed T-bet on IFN-γ promoter activity and can be tested *in vitro* by EMSA or by ChIP assays. This will enable the functional interaction of T-bet protein with IFN-γ promoter construct to be determined. Indeed, the current findings clearly indicate that the effect of TGF-β1 on early IFN-γ production (3 hours) occurs independent of T-bet expression.

^{612}GTGGTCTGCCT^{602}.....CAAA^{263}TGGTGTGAAG^{254}TAAAAGTGCTTTC
AGAGAATCCCACAAGAATGGCACAGGTGGGCACAGCGGG^{202}GCTGTC
TCATCGTCAGAGAGCCCAAGGAGTCGAAAGGAAACTCTAACATGCCA
CAAAACCATAGCTGTAATGCAAAGTAACTTAGCTCCCCCCACCT^{104}AT
CTGTCACC^{95}ATCTTAAAAAAAAAAAAAAACCAAAAAAAA^{66}ACTTGTG
AAA^{57}ATACGTAATCCCGAGGAGCCTTCGATCAGG^{27}TATAAA

Figure 5.10: Partial sequence of the mouse IFN-γ promoter is shown:
Three characterised T-bet binding sites (TGTGAA/TGTCAC) are highlighted green (J. Y. Cho *Et. al.*, 2003). Two potential SMAD binding sites (GTCT) are highlighted in yellow (Yu *Et. al.*, 2006). Superscript numbers indicate the positions of nucleotides in sequence to transcription start site (TSS, highlighted in red) of the mouse gene (Penix *Et. al.*, 1993).

While the current research was ongoing, Yu *Et. al.* proposed two molecular mechanisms for TGF-β1 induced IFN-γ suppression in human NK cells via Smad proteins, one of which was T-bet-dependent and other was T-bet-independent (Yu *Et. al.*, 2006). From the investigations to date (recent reviews by Marcais *Et. al.*, 2013; Sanjabi *Et. al.*, 2017), this is the only study conducted that is closely-related and their findings remain relevant to the current study. It is thus essential to understand the key differences between the current study and Yu *Et. al*'s study. They showed that, on 24 hours of stimulation with IL-12 plus IL-18 in the presence of TGF-β1, IFN-γ production was inhibited either directly via activated Smad-3 or indirectly by Smad-mediated down-regulation of T-bet expression (Yu *Et. al.*, 2006). In-line with this,

another study also demonstrated a T-bet-dependent pathway as the underlying mechanism in TGF-β1-mediated IFN-γ suppression (Koutoulaki *Et. al.*, 2010). Koutoulaki *Et. al.* showed that TGF-β1 treatment of DCs stimulated with IL-18 for 24 hours resulted in suppression of IL-18-up-regulated T-bet expression to constitutive levels in IFN-γ production. In the current study, neither stimulation with IL-12 resulted in increased T-bet expression nor was TGF-β1 able to down-regulate constitutively expressed T-bet levels. Thus, this rules out the proposed T-bet-dependent regulation of IFN-γ by TGF-β1 in KY-1 cells.

An important difference between the current study and Yu *Et. al*'s study was the time of stimulation (3-6 hours vs 24 hours) with IL-12 in the presence of TGF-β1. This is significant with regards to the induction of T-bet. Perhaps, in the current study, a short-term stimulation with IL-12 was adequate for rapid IFN-γ production but might not be sufficient to induce T-bet expression. Other studies have reported using T-bet$^{-/-}$ NK cells that early and rapid production of IFN-γ is T-bet independent (6 hours). However, after 24 hours of cytokine stimulation, IFN-γ production was found to be significantly reduced in the absence of T-bet (Szabo *Et. al.*, 2002; Townsend *Et. al.*, 2004). This suggested the importance of this factor in the later production and maintenance of IFN-γ levels (Way & Wilson, 2004; Leong *Et. al.*, 2017). In this context, Matsuda *Et. al.* (2007) demonstrated using an ectopically inducible form of T-bet in CD4^{+} T cells that early induction and nuclear translocation of T-bet occurred in 2 hours of stimulation by a synthetic drug. Yet, the T-bet-mediated effect on IFN-γ production is delayed and required between 12 to 24 hours. Similarly, naïve CD4^{+} T cells on TCR stimulation showed T bet expression in 1 hour but the effector cytokine production was not seen until 48 hours (Yu, Wu *Et. al.*, 2014). Thus, the current findings in conjunction with Yu et al's findings raises an intriguing possibility in temporal control of IFN-γ expression by TGF-β1 via T-bet. Because of the short-term activation time used in the current study, T-bet induction by IL-12 and its inhibition by TGF-β1 that could occur at later time points was not observed. However, to address this, studies with kinetics of T-bet expression on IL-12 stimulation in the presence and absence of TGF-β1 will be required as future work.

Another mechanism proposed by Yu *Et. al.* was the direct regulation of IFN-γ expression by Smads (T-bet independent pathway). This pathway was shown to be mediated through inhibition of IFN-γ promoter activity, in the proximal region

spanning -204 bp from TSS of IFN-γ gene by Smad proteins (Smad 3 and 4). Further, by electrophoretic mobility shift assay (EMSA), they demonstrated direct binding of Smad-3 to distinct regions on the IFN-γ promoter (-204 to -138 bp), called Smad binding elements (SBE) (Yu *Et. al.*, 2006). However, the current study demonstrated that TGF-β1 did not affect IL-12-induced IFN-γ promoter activity, within a -777bp to +48bp promoter sequence that was transiently expressed in NK cells (Figure 5.9A). Even though, as determined through bioinformatic analysis, this region of the promoter contained two SBE (GTCT), as shown in Figure 5.10 (yellow region). Thus, in contrast to the proposed Smad-mediated model, the current findings suggest that the molecular basis to early IFN-γ suppression observed was not due to direct binding of TGF-β1-activated Smads to IFN-γ promoter. Hence, the proposed T-bet independent pathway by Yu et al seems unlikely as a possible mechanism in our case. One important point of distinction to note is the difference in species. Yu *Et. al.*'s study was performed using a human IFN-γ promoter construct with a sequence between -204 and -138bp, that contained three consensus Smad-binding motifs, with two sites adjacent to each other and a third one in close proximity. Whereas, in the current study, the murine counterpart contained only one motif within the specified region which may not be sufficient to exhibit direct Smad-mediated IFN-γ promoter repression and hence, observed lack of TGF-β1 effect on IFN-γ promoter activity.

It is also worth noting that, in the above-mentioned Yu *Et. al.* study, the approach used to demonstrate the T-bet-dependent and independent mechanism via Smads in IFN-γ suppression was overexpression of specific Smads or T-bet proteins respectively, the effects of which warrants careful consideration. Overexpression of these proteins do not reflect the physiological or endogenous signalling pathway activated by specific stimuli. For instance, the key events in TGF-β1-mediated signalling are phosphorylation and association of specific Smads with each other. It was evident from some of the Yu *Et. al*'s results that modest IFN-γ inhibition with overexpressed Smad-3 proteins was further enhanced in the presence of TGF-β1 (Yu *Et. al.*, 2006). Moreover, phosphorylation of transcription factors results in their transition between cellular compartments during a signal transduction pathway (N. S. Chang *Et. al.*, 2005). Trotta and colleagues showed using a specific inhibitor of TGF-β1 receptor kinase (SB431542) in NK cells that nuclear phosphorylation of Smad-3 is necessary for the inhibition of IFN-γ by TGF-β1. They further showed that the difference in the levels of phosphorylation of overexpressed Smad-3 proteins in the nucleus, in the presence and

absence of TGF-β1, reflected in the differential inhibition of IFN-γ production (Trotta *Et. al.*, 2008). Likewise, ectopic expression of T-bet has been known to up-regulate endogenous T-bet levels that functions to induce histone modifications and chromatin remodelling events in IFN-γ expression, even in the absence of STAT-4 or IL-12 signalling (Mullen *Et. al.*, 2001; Shnyreva *Et. al.*, 2004; Tong *Et. al.*, 2005).

Nevertheless, from these studies, it seems there are multiple mechanisms in TGF-β1-mediated IFN-γ suppression as the subsequent findings from Yu *Et. al*'s group have shown that Smad-3$^{-/-}$ and T-bet$^{-/-}$ NK cells exhibited IFN-γ production that was partially and strongly inhibited by TGF-β1 (Trotta *Et. al.*, 2008). Thus far, the mechanism and the mediators of this are still unknown. In fact, consistent with our findings, a study from another group of investigators demonstrated that TGF-β treatment of NK cells did not alter T-bet or STAT-4 mRNA expression but resulted in an increased expression of transcripts for a transcription factor, GATA-3 (Lindgren at al., 2011). GATA-3, a Th2 transcription factor is known to inhibit IFN-γ production (Kaminuma *Et. al.*, 2004). Lindgren et al also noted a similar trend in expression of transcripts for these transcription factors (T-bet, STAT-4 and GATA-3) in NK cells from cancer patients supporting the role of tumour-derived TGF-β in the suppression of IFN-γ. Their findings suggested a possible role of GATA-3 in TGF-β-mediated suppression of IFN-γ (Lindgren at al., 2011). However due to the lack of data on whether T-bet or GATA-3 proteins are expressed, this hypothesis requires further investigation to establish a definitive association between GATA-3 and TGF-β. Hence, the results from the current study alludes to existence of an alternative mechanism in the early and rapid suppression of IL-12-signaled IFN-γ by TGF-β1 within hours.

It is still important to understand, by what mechanism TGF-β1 inhibits IFN-γ production within hours? One possible scenario could be that TGF-β1 might induce chromatin remodeling in transcriptional repression of the IFN-γ gene in NK cells. In fact, the current results that demonstrates IFN-γ production within hours of IL-12 stimulation (as seen in Figure 5.4C), also correlates with the 'open' status of *Ifng* locus in NK cells (Tato *Et. al.*, 2004). This 'open' status in NK cells refers to a constitutively modified form of chromatin at *Ifng* locus such as histone acetylation and demethylation that permits accessibility to transcriptional machinery for IFN-γ production, whereas this status is acquired in T cells through cell cycle progression and proliferation (Tato *Et. al.*, 2004; Tato *Et. al.*, 2006; Aune *Et. al.*, 2013; Cockerill, 2016). This is consistent

with previous reports which had demonstrated in ChIP assays using antibodies to acetylated H3 that *Ifng* locus in NK cells is highly acetylated around the IFN-γ promoter. Moreover, these levels of H3 acetylation (an indicator of accessible chromatin) in resting NK cells matched to the levels in differentiated $CD4^+$ T cells, whereas naive $CD4^+$ and $CD8^+$ T cells expressed low levels of histone acetylation (Stetson *Et. al.*, 2003; Tato *Et. al.*, 2004). This indicated that, unlike in T cells, the derepressed *Ifng* locus in NK cells is apparently accessible to transcription factors, which in turn, allows immediate production of IFN-γ on cytokine stimulation.

However, it is also known that besides the constitutive acetylation patterns in resting NK cells, these cells additionally acquire histone acetylation patterns at certain regions of the IFN-γ gene depending on the specific cytokine (IL-12/IL-12+IL-18) signalling pathway that activates IFN-γ gene transcription. For instance, stimulation of resting NK cells with IL-12 for 6 hours resulted in a specific increase in H4 histone acetylation levels at -6kb 5' of the IFN-γ gene to a similar extent as Th1 cells but did not modify other acetylated or non-acetylated regions of the IFN-γ gene (S. Chang & Aune, 2005). IL-12-activated STAT-4 was shown to alter the chromatin microenvironment of the *Ifng* locus in Th1 cells by inducing histone hyperacetylation and recruiting chromatin associated enzymes (Thieu *Et. al.*, 2008; Balasubramani *Et. al.*, 2014). Thus, even though the *Ifng* locus in NK cells is in a permissive state for rapid transcription, it undergoes chromatin remodelling on stimulation with IL-2/IL-12 (Bream *Et. al.*, 2004), IL-12/IL-18 (S. Chang & Aune, 2005) that induces active IFN-γ gene transcription.

Consistent with this view, it is interesting to note that TGF-β-activated Smad complexes have shown to directly recruit chromatin-modifying proteins or Smad-associated repressor proteins in transcriptional repression (as recently reviewed by Hata & Chen, 2016; Hill, 2016). For example, phosphorylated Smad-3 has been shown to recruit HDAC 4/5 in repression of TGF-β regulated gene expression (J. S. Kang *Et. al.*, 2005). The recruitment of HDACs reverses histone modifications by removing histone acetylation mediated by activating factors or induces repressive histone marks that results in limited accessibility of the DNA template to transcription factors (Wolffe *Et. al.*, 2000). Transcriptional repression can also be achieved by Smads in association with co-repressor proteins such as TG3-interacting factor (TGIF). TGIF bridges Smad complexes (Smad-2/4) with chromatin condensing histone deacetylases (HDACs) onto specific TGF-β responsive target genes (Wotton *Et. al.*, 1999a; Wotton *Et. al.*, 1999b).

Therefore, based on such direct interaction of TGF-β-activated Smads with chromatin-modifying proteins or co-repressors in repression of gene transcription, one could further hypothesize that the potential mechanism responsible for TGF-β1-mediated IFN-γ inhibition might be through epigenetic regulation that is chromatin remodeling of the *Ifng* locus.

Interestingly, in line with this proposition, research work in the field studying an integrated signal from cytokines, TGF-β1 and IL-6 has shown to converge at the chromatin level within 4 hours, which regulated the functional activities of FOXP3, a specific transcriptional repressor in T_{reg} cells. Expression and acetylation of FOXP3 is associated with suppressive functions of T_{reg} cells in autoimmunity or pro-inflammatory conditions. TGF-β1 alone treatment resulted in increased acetylated levels of FOXP3 and in turn enhanced chromatin binding that lead to repression of the IL-2 promoter. While IL-6 signalling in the presence of TGF-β1 reduced chromatin binding of FOXP3, a process that was shown to be reversed by a HDAC inhibitor, sodium butyrate (Samanta *Et. al.*, 2008). Thus, a cross-talk between TGF-β1 and IL-12 signalling in IFN-γ production might be at the chromatin level. Since, the general view in the field suggests that histone acetylation of promoter precedes active transcription, changes in acetylation might reflect to alterations at transcription level (Narlikar *Et. al.*, 2002; Hill, 2016). Therefore, to explore this possibility, the acetylation status of *Ifng* locus in the presence or absence of TGF-β1, using quantitative ChIP assays and anti-acetyl histone H3/H4 antibodies could be determined in the future work.

Consistent with many other studies (Bellone *Et. al.*, 1995; Bright & Sriram, 1998; Sudarshan *Et. al.*, 1999; Hayashi *Et. al.*, 2003; Yu *Et. al.*, 2006; Trotta *Et. al.*, 2008; Koutoulaki *Et. al.*, 2010), the current study observed only a partial decrease in IFN-γ produced by NK cells in the presence of TGF-β1 but the *in vivo* consequences for NK cell-dependent responses may be profound. For example, TLR9-deficient mice exhibited only 25–30% reduction in NK cell-produced IFN-γ (Krug *Et. al.*, 2004). Despite this partial reduction in IFN-γ production, the ability of TLR9-deficient mice to clear murine cytomegalovirus (MCMV) infection was significantly impaired in comparison with wild-type (WT) mice (Krug *Et. al.*, 2004). Moreover, as implied from our studies and that of other investigators in the field (recent reviews by Marcais *Et. al.*, 2013; Zwirner & Ziblat, 2017; X. Ma *Et. al.*, 2015; Sanjabi *Et. al.*, 2017) that the

degree of NK cell activation is determined by the balance of inhibitory (TGF-β1/ IL-10) and stimulatory (IL-12/ IL-2/ IL-15/ IL-18) cytokines present in the microenvironment, significantly in a tumour microenvironment. Therefore, information from this study on cytokine signalling cross-talk could help design strategies in the management of cell-mediated immune responses in individuals, most importantly immunocompromised individuals, including cancer patients, against various infections and diseases.

CHAPTER 6

GENERAL DISCUSSION AND OUTLOOK

The overall aim of this thesis was to enhance the mechanistic understanding of NK cell activity and thereby regulating the balance of Th1 /Th2 type immune responses through the role of cytokines and complex carbohydrates such as GAGs/PGs. This thesis sought to elucidate a molecular understanding of cytokine binding to heparin/HS, and regulation of IL-12 signalling-induced IFN-γ production in NK cells by DS-PG and TGF-β1.

The specific goals of this research were:

1. To examine three interleukins from different families of cytokines, for their heparin-binding property through identification of putative heparin binding domains within their primary, secondary and/or tertiary structures of IL-11, IL-18 and IL-22 (Chapter 3).
2. To investigate the underlying mechanism by which DS-PGs might participate in the induction of IFN-γ secretion (Chapter 4).
3. To analyse molecular mechanisms underlying the inhibitory effects of TGF-β1 on IL-12 induced IFN-γ expression and secretion (Chapter 5).

The following sections summarise the key findings for each of these goals, discuss their implications, and the ideas for future work.

6.1 Binding Studies of IL-11, IL-18 and IL-22 to Heparin/HS

To the best of our knowledge, to date, the results presented in Chapter 3 are the first to demonstrate that interleukins 11, 18, and 22 do not bind to heparin at physiological pH and ionic strength. This comprehensive research work integrated structural and experimental approaches to screen and determine heparin-binding characteristics on each of three interleukins. The results confirm that these interleukins do not share the heparin-binding property with their respective family members, despite sharing a pronounced protein structural fold. On comparing each of these interleukins, IL-11, IL-18 and IL-22 with their heparin-binding counterparts, one of the key findings is that the presence and position of acidic amino acids pose unique challenges in the formation of stable heparin-binding domain. Our results also parallel that of TGF-β3 which does not bind to heparin/HS, while other isoforms (TGF-β1 & TGF-β2) within the TGF-β family do bind (Lyon *Et. al.*, 1997; J. Lee *Et. al.*, 2015). This example further affirms

our key finding that each cytokine of interest needs to be studied individually when seeking to determine its heparin-binding characteristics.

Current findings in Chapter 3 led to a discussion in understanding the structural basis of non-heparin-binding characteristic of interleukins 11, 18 and 22 compared to their respective heparin-binding family members. As mentioned previously in Chapter 1 (Section 1.5) that, in the event of negative findings, in which case, the selected interleukins do not bind to heparin/HS, this may still have significant biological implications as discussed here. For IL-11 and IL-22, the lack of affinity for heparin may be a desirable property that enables these interleukins to remain unbound and free. Once secreted, these interleukins may travel freely through circulation to reach their receptors on remote target organs. This theory could be supported knowing that IL-11 mainly functions in the development and maturation of myeloid and lymphoid cells during haemopoiesis in the bone marrow but also plays a role in many other distant tissues such as brain, gut, testis and bone (Du & Williams, 1997; Sims *Et. al.*, 2005). Interestingly, recent findings provide evidence for the role of IL-11 in breast cancer metastasis to distant sites, particularly to bone (Johnstone *Et. al.*, 2015). Indeed, IL-11 with osteotrophic properties is a key regulator in bone metabolism through differentiation of osteoblasts into osteoclasts by binding to their cell surface receptors (Sims *Et. al.*, 2005). One mechanism suggested in this osteolytic metastasis was increased osteoclast activation by tumour microenvironment secreted IL-11 (Kozlow & Guise, 2005). In this context, ectopic expression of IL-11 in colorectal cancer cells was shown to result in enhanced metastasis to multiple organs compared to minimal metastasis observed by ectopically expressed IL-6, a heparin-binding counterpart of IL-11 (Calon *Et. al.*, 2012). Given the recently known role of IL-11 in various cancer types (Hanavadi *Et. al.*, 2006; Putoczki *Et. al.*, 2013; Negahdaripour *Et. al.*, 2016) and in the light of current findings, one could attribute some of the endocrine effects of IL-11 over distant sites to its non-heparin-binding property, however this correlation remains to be established in future investigations.

Similarly, IL-22 produced by immune cells at the site of local inflammation has actions on non-immune cells. This functional effect remarkably distinguishes IL-22 from other members of IL-10 family of cytokines (Witte *Et. al.*, 2010; Dudakov *Et. al.*, 2015). Functional IL-22 receptors are present on the epithelial cells and fibroblasts of various tissues/organs such as skin, gut, liver, thymus, pancreas and lungs, which thus become

targets for this cytokine, resulting in innate, non-specific immunity in these far-off tissues (Wolk *Et. al.*, 2004; Sabat *Et. al.*, 2014). In this context, following local tissue inflammation, the presence of IL-22 has been detected in the circulation (Wolk *Et. al.*, 2006a; Wolk *Et. al.*, 2007; Pan *Et. al.*, 2013). For example, crohn's disease (CD), a chronic inflammatory bowel disease which is characterised by infiltration of activated immune cells in the intestine, results in secretion of IL-22 into the circulation. This systemic IL-22 was shown to exhibit a protective role by inducing production of anti-bacterial proteins by hepatocytes into the blood, thus preventing systemic inflammation in CD patients (Wolk *Et. al.*, 2007). This example clearly indicates a possible endocrine role of IL-22. Moreover, independent expression of IL-22-specific signalling receptor, IL-22R1 on diverse and distant tissues further support the systemic function of IL-22 (Sabat *Et. al.*, 2014). Collectively, these instances suggest the effects of IL-11 or IL-22 over longer distances through diffusion into blood circulation. Thus, selectivity of cytokines in not binding to heparin and its influence on a biological process can be the focus of future studies.

IL-18 has been observed as a biomarker in patients with metabolic syndrome, in which many organs and tissues such as adipose, muscle tissues secrete IL-18, resulting in elevated levels in the circulation (Troseid *Et. al.*, 2010). The circulating high levels of this cytokine was shown to play a crucial role in the development of type 2 diabetes mellitus and cardiovascular diseases (Hivert *Et. al.*, 2009). It is thus, plausible to suggest from these observations that IL-18 can freely diffuse from the tissue microenvironment or matrix into blood circulation to cause these pathogenic effects in patients with metabolic syndrome. Very recently many cytokines and growth factors including some members of FGF family have been reported to exhibit non-heparin-binding properties that allows diffusion and free flow in circulation (Itoh *Et. al.*, 2015; Nunes *Et. al.*, 2016), thus activating distant receptors in physiological events or off-targets in pathophysiological roles. Based on the data presented here that IL-18 does not bind to heparin/HS, one could possibly explain the free diffusion of secreted IL-18 and its effects through the blood circulation. However, this association remains to be determined for IL-18 until now, whereas it is known for other cytokines like FGFs-15, -19, -21, -23.

Strikingly, FGFs-15, -19, -21, -23, the new members of the classical FGF family, despite of sharing a common β-trefoil structural motif with rest of the heparin-binding,

FGF family members exhibited weak or no affinity for heparin (Goetz *Et. al.*, 2007). This group of non-heparin binding FGFs belong to the FGF-19 sub-family and are referred to as endocrine FGFs based on their systemic mode of action controlling various metabolic processes (Itoh *Et. al.*, 2015; Nunes *Et. al.*, 2016). The contrast in the heparin-binding property between the endocrine FGFs from the rest of the paracrine FGF family members existed due to the unique conformation adopted by the regions/loops within the β-trefoil scaffold, as revealed by superimposition of their crystallised structures (Goetz *Et. al.*, 2007), while the heparin-binding FGF members such as FGF-2 exhibited a HBS, a composite basic site contributed by the loop regions of β strands, β10 and 11 forming a surface groove as seen in Figure 3.18. Moreover, the basic residue at the base and those surrounding the groove enabled a stable and stronger interaction with heparin/HS (L. D. Thompson, 1994; Faham *Et. al.*, 1996; Raman *Et. al.*, 2003; R. Xu, *Et. al.*, 2012). On the other hand, Goetz et al revealed that HBS in members of the FGF-19 subfamily possessed a positively-charged, surface crest or ridge, formed by the loop regions of β1-2 strands and components of β10 and 12 strands. Additionally, these narrow surface structures were reported to be sterically weak to support HS binding (Goetz *Et. al.*, 2007; Goetz & Mohammadi, 2013).

FGF family members are considered prototypical heparin-binding proteins with an absolute requirement of heparin/HS for their biological function (Yayon, 1991; Rapraeger *Et. al.*, 1991). Indeed, much of the thinking in the field has been stimulated by the FGF-heparin story. Therefore, these findings by Goetz et al in 2007 demonstrated diversification within the FGF family with regards to heparin/HS interaction. This has also staged the intriguing research on non-GAG-binding cytokines and their biological significance at the forefront in the field of matrix biology. As proposed for these specific endocrine FGF proteins, the lack of affinity for heparin enables them to be more mobile in the extracellular and intravenous environment. Whilst, remaining unbound to tissue HS, these proteins are available to distant target organs, and therefore exhibit an endocrine mode of action. For example, FGF-19 is produced in the intestine but acts on liver regulating bile synthesis and cholesterol metabolism (Wu *Et. al.*, 2007). Similarly, FGF-23 is produced in bone and acts on receptors expressed by kidney cells regulating calcium absorption and vitamin D synthesis (Kurosu *Et. al.*, 2006). This is in contrast to paracrine/autocrine mode of activity used by the majority of heparin-binding members of FGF family to exert their biological effects, close to their site of secretion (Asada *Et. al.*, 2009; Kuro-o, 2012;

Itoh *Et. al.*, 2015; Nunes *Et. al.*, 2016). Analogous to these examples of FGF-19 subfamily members, which have a role in regulation of metabolic activities, in the case of IL-11, IL-18 and IL-22, the non-heparin binding property maybe of additional benefit to these cytokines to act in an endocrine mode to fulfil some of their biological functions, as described earlier.

In fact, findings have confirmed that the FGF-19 subfamily members are required to bind to special receptors called α or β klotho proteins expressed on the target tissues to signal. Binding to these receptors substitute the need of binding to heparin/HS-GAGs for signalling. Similar to the role played by heparin/HS in FGF signalling, these klotho proteins act as co-receptors for endocrine FGFS, as they can bind co-operatively to both the ligand (FGFs) and their respective FGFRs (Beenken & Mohammadi, 2009; Kuro-o, 2012; Belov & Mohammadi, 2013). Structural studies are awaited, determining the crystal structure of ternary complexes by endocrine FGF, FGFR and klotho receptors (Goetz & Mohammadi, 2013). Since, these klotho co-receptors were found co-expressed with paracrine FGFs (Urakawa *Et. al.*, 2006), it has now been established that, by binding to FGFRs, these Klotho receptors play a dual role in inhibiting paracrine FGF signalling and concurrently enhancing the specificity of FGFRs for endocrine FGF ligands (Goetz *Et. al.*, 2012a; Goetz *Et. al.*, 2012b). Among widely expressed FGFRs, tissue-specific expression of klotho proteins allows these endocrine FGFs to determine their target organs and hence define specificity in FGF-FGFR interaction (Kuro-o, 2012; Itoh *Et. al.*, 2015). The role of klotho receptors and endocrine FGFs is an intriguing story and is still unfolding. Based on recent reviews, efforts are presently being directed in understanding the molecular basis of endocrine FGF signalling complex formation as compared to paracrine FGF signalling (Itoh *Et. al.*, 2015; Nunes *Et. al.*, 2016; Sun *Et. al.*, 2016). Similar information on the biological relevance of other non-GAG-binding cytokines is lacking. Nonetheless, non-GAG-binding cytokines are now attracting considerable interest in this field of research.

The subject of GAG-binding and bioavailability may be particularly important in the evaluation of cytokines for potential therapeutic use. In some circumstances, it may be more effective to employ a non-heparin binding than a heparin-binding cytokine. For example, IL-18 functionally resembles IL-12 *in vitro* and *in-vivo* (Trinchieri *Et. al.*, 2003). The recombinant forms of both cytokines have been exploited as vaccine adjuvants in anti-tumour therapy (Rodriguez-Galan *Et. al.*, 2009). However, systemic

administration of rIL-12 has been known to be associated with dose- and schedule-dependent toxicity (Rook *Et. al.*, 2001). Toxicity is related to the combined activity of both the cytokines, IL-12 and the induced IFN-γ, (J. P. Leonard *Et. al.*, 1997; Lenzi *Et. al.*, 2007). Interestingly, both these cytokines are known heparin-binding proteins (Lortat-Jacob *Et. al.*, 1991b; Hasan *Et. al.*, 1999). Indeed, heparin-bound IFN-γ has a markedly increased biological half-life in the circulatory system (from 1.1 to 99 mins) (Lortat-Jacob *Et. al.*, 1996a). Therefore, persistently high serum IL-12 and IFN-γ concentrations may explain the toxicity associated with systemic administration of rIL-12. On the other hand, IL-18 is a weaker inducer of IFN-γ than IL-12 (Yoshimoto *Et. al.*, 1998). The serum IFN-γ concentration in cancer patients receiving rIL-18 was found to be substantially lower than that observed on rIL-12 administration (Robertson *Et. al.*, 1999). Moreover, as demonstrated in the current study that IL-18 does not bind heparin, this protein may therefore, be retained for less time in the circulatory system. This in part, may explain the mild toxicity associated with IL-18 administration. This possibly also explains why threefold higher doses of rIL-18 could be administered than doses of rIL-12 (J. P. Leonard *Et. al.*, 1997). Hence, the non-heparin-binding property may present an advantage in the clinical development of rIL-18 monotherapy over rIL-12 for systemic cancers.

This is also true in the case of IL-11 and IL-6 which have overlapping functional properties (Musashi *Et. al.*, 1991; Negahdaripour *Et. al.*, 2016). These cytokines have potential compensatory roles due to a common receptor-subunit, gp130-associated signalling pathway (Dahmen *Et. al.*, 1998; Barton *Et. al.*, 2000). However, IL-6 is a well-characterised, heparin-binding equivalent of IL-11 (Mummery & Rider, 2000). When rIL-6 was administered as therapeutic agent in the management of haematologic disorders, such as thrombocytopenia due to myelodysplastic syndromes (MDS), it was reported with only low to modest thrombopoietic activity but a significant dose-limiting toxicity (M. S. Gordon *Et. al.*, 1995). Whilst, when the role of IL-11 as thrombopoietic growth factor was investigated in a clinical setting (Goldman, 1995; M. S. Gordon, 1999), this cytokine was successful at low dose in patients with bone marrow failure (BMF)-associated thrombocytopenia due to MDS (Tsimberidou *Et. al.*, 2005). Further, the clinical use of IL-11 was extended as an effective treatment in chemotherapy-induced thrombocytopenia in malignant patients (Bhatia *Et. al.*, 2007). However, the observed reduced efficacy of rIL-6 in the clinical setting could be well understood based on it's heparin-binding property. The interaction of IL-6 with heparin

was shown to reduce the availability of this cytokine ligand to its receptor complex (Mummery & Rider, 2000). Supporting this, use of heparin mimetics such as suramin not only blocked IL-6 binding to its cell-surface receptors but also inhibited its biological activity (Strassmann *Et. al.*, 1993). On the other hand, the presence of heparin was shown to synergistically and mechanically augment IL-11 activity (Walton *Et. al.*, 2002; Rajgopal *Et. al.*, 2006). This effect, coupled with the current results demonstrating that IL-11 does not bind to heparin/HS, possibly explains the potential use of IL-11 as a therapeutic intervention in place of IL-6. Moreover, this substitution in clinical application is gaining particular attention with the emerging role of IL-11 in GI cancers over IL-6 (Putoczki *Et. al.*, 2013; Negahdaripour *Et. al.*, 2016). The data presented here highlights an important difference affecting the functional mechanism of IL-11 compared to IL-6 that will be useful in improving the therapeutic efficacy of IL-11 and its recombinant variants. Collectively, in consideration of our results, the chosen group of cytokines, IL-11, IL-18 and IL-22 can be evaluated for efficacy in future clinical trials as single agents, or in adjuvant combinations with other cytokines or drugs.

Whilst the current investigation was underway, there have been no additional publications on binding of IL-11, IL-18 and IL-22 to heparin/HS. Therefore, these interleukins could be added to a relatively small group of known non GAG-binding cytokines, such as IL-9, IL-16, and TNF-α (Rickard *Et. al.*, 2003). GAG-protein interactions are a long standing research area and remarkable progress has been made in this field of matrix biology to identify heparin-binding proteins (HBPs) and to gain additional insights into such interactions. Interestingly, chemokine CXCL11 was demonstrated to hold two different affinity levels for heparin, suggesting 'structural plasticity' in its protein conformation that enabled such a heterogenous heparin-binding property (Severin *Et. al.*, 2010). In addition to this example of CXCL11, the non-heparin-binding members from FGF family as described above shows that the interactions between cytokines and GAGs are more diverse than previously understood. Thus, the findings from the current research described in Chapter 3, along with recent work in the field have contributed to the changing view that demonstrates the existence of diversity in cytokine-heparin/HS interactions within the same class of cytokines (recent reviews by Itoh *Et. al.*, 2015; Nunes *Et. al.*, 2016; Y. Li *Et. al.*, 2016; Rajarathnam *Et. al.*, 2018). Indeed, this is consistent with other studies that a GAG-binding property maybe an additional feature to the subset of cytokine family members

(Coombe, 2008; Dyer *Et. al.*, 2016). Therefore, the current work re-emphasizes the importance of knowing whether GAG binding is a requisite to the function of the cytokine or whether GAG binding ability is only an added advantage to cytokine function.

With regards to the future work in heparin-binding studies, IL-23 may be proposed as a likely candidate. IL-23 is a more recently discovered member of the IL-12 family of heterodimeric cytokines that also shares the heparin-binding subunit, p40 of IL-12 (Oppmann *Et. al.*, 2000; Vignali & Kuchroo, 2012). Like IL-12, IL-23 is primarily produced by antigen presenting cells, macrophages and dendritic cells. Both IL-12 and IL-23 have overlapping functions of inducing lymphocyte (NK and T cells) proliferation and IFN-γ production, thus a role in cellular immunity and inflammation (Langrish *Et. al.*, 2004; Duvallet *Et. al.*, 2011; Teng *Et. al.*, 2015). Moreover, IL-23 drives development of Th17 cells that is central to the development of chronic autoimmune diseases (Langrish *Et. al.*, 2005; Lubberts, 2015). Therefore, both IL-12 and IL-23 are attractive therapeutic targets for various autoimmune diseases (as reviewed in Floss *Et. al.*, 2015). Nonetheless, IL-23 has been shown to contribute largely to local inflammatory responses in contrast to the systemic inflammation induced by IL-12. This differential role of IL-12 and IL-23 was demonstrated in the murine model of inflammatory bowel diseases (IBDs), which indicated that IL-23 could be a selective drug target in IBDs (Uhlig *Et. al.*, 2006; Teng *Et. al.*, 2015). The proposed goal in determining the heparin-binding ability of IL-23, or lack thereof, would likely be used to selectively target the cytokine and modulate its response.

Structurally, IL-23 is a combination of p40 subunit of IL-12 with p19 subunit. Though p40 subunits, besides forming the heterodimers with p19 and p35 subunits, which are distantly related, they are also known to form homodimers. These p40 homodimers have biological activity antagonistic to IL-12 (Gillessen *Et. al.*, 1995; Trinchieri & Scott, 1995a) and IL-23 (Shimozato *Et. al.*, 2006). However, IL-12 p40 deficient mice showed impaired antigen-stimulated IFN-γ production and Th1 responsers (Magram *Et. al.*, 1996). With this regulatory role, p40 proteins are also found secreted in excess over the p70 heterodimers of IL-12 in many disease states (Abdi *Et. al.*, 2014). IL-23 binding via the p40 subunit to heparin/HS of the ECM is functionally significant as p40 is a target protein in number of autoimmune and chronic inflammatory diseases (review in Croxford *Et. al.*, 2014). Moreover, Ustekinumab, a p40 specific monoclonal

antibody that blocks IL-12/IL-23 signalling (Benson *Et. al.*, 2011) is an approved therapeutic treatment for psoriasis (Yielding *Et. al.*, 2012).

Interestingly, the heparin-binding property of IL-12 was shown being largely located in the larger subunit, p40 rather than its smaller subunit p35 that is involved in IL-12-receptor interactions (Jayanthi *Et. al.*, 2017; Garnier *Et. al.*, 2018). Moreover, previous studies from our laboratory have shown that the p40 subunit by itself exhibited strong binding affinity like IL-12 to heparin /HS (Hasan *Et. al.*, 1999). Importantly, recent studies have extensively characterized this interaction using chemically modified heparins as competitive inhibitors and theoretical docking calculations to locate basic charge clusters involved in heparin binding (Garnier *Et. al.*, 2018). They have shown that the heparin binding site on IL-12 and a homodimer of its p40 subunit consists of a cluster of six basic amino acids at the tip of C'D' loop within the D3 domain of its carboxy-terminus, as shown in Figure 6.1. Moreover, this cluster is analogous to the carboxy-terminal cluster of 4-5 basic residues as observed in IFN-γ (Lortat-Jacob & Grimaud, 1991a), IL-8 (Webb *Et. al.*, 1993) and CXC chemokines (Witt & Lander, 1994) that confers the heparin-binding property.

p40:

^{256} QGKSKREKKDRVF ^{268}

Figure 6.1: Primary sequences of putative heparin/ HS binding binding site in human p40 showing basic amino acids:
The sequence of mature, secreted form of human p40 with basic amino acids shown as coloured letters, arginine = **R** and lysine = **K**. All amino acid residues are represented by one letter code. The sequence is taken from (Wolf *Et. al.*, 1991) with superscript numbers referring to amino acid numbering in the secreted polypeptide.

According to another study which conducted a comparative structural analysis of interfaces formed by the p40 subunit with p19 and p35 subunits respectively, revealed that the p40 subunit uses the same binding surface and the majority of contact residues are common in binding independently with p19 and p35 subunits (Lupardus & Garcia, 2008). In fact, this same study showed that p40 conserves a structural 'hotspot', critical in the formation of heterodimeric complex in IL-12 as well as IL-23. However, when the p19 subunit of IL-23 engages with p40, there are substantial changes with respect

to the docking modes on p40, which are structurally distinct from the interaction by the p35 subunit of IL-12. This was revealed by superimposition of the p40 subunit from both complexes of respective cytokines (IL-12 and IL-23) highlighting the structural differences and contact residues (Lupardus & Garcia, 2008). Thus, an investigation of whether p40 retains the heparin-binding property while combining with p19 to form IL-23 is justified. Since p40 itself binds to heparin/HS and retains this property when in combination with p35 to form IL-12, the likelihood remains that IL-23 may also possess this binding property. Nevertheless, as highlighted by the current findings, there are various factors that influence the formation of a heparin-binding domain in protein based on its three-dimensional structure. Indeed, the p40 subunit contains a heparin-binding sequence, but the structural interaction of this subunit with another subunit-p19 with spatial orientation of basic residue clusters will determine whether IL-23 binds to heparin/HS or not. As proposed in the case of IL-12, that the heparin-binding site was conformationally formed of basic residues from unordered and flexible loop regions within the D3 domain of the C-termini, any conformational change can affect the availability of exposed residues for interaction with heparin. The crystal structure of IL-23 is available in the PDB (PDB ID: 3DUH) (Lupardus & Garcia, 2008). Very recently, a high-resolution structure of the IL-23 in complex with its cognate receptor, IL-23R has been generated (Bloch *Et. al.*, 2018). They have shown that the receptor chain mainly engages with the exposed end of the p19 subunit. In this receptor interaction there is also a minor contact with an aminoterminal domain, the D1-D2 region of the p40 subunit which slightly influences carboxy-terminal domain D3 orientation, but the surface of the p40 domain, D3 remains largely exposed. This suggests an increased likelihood of IL-23 to be able to bind heparin/HS via the p40 subunit. Using a strategy of combined structural and experimental approaches as employed in the current study, one can investigate heparin-binding property of IL-23.

6.2 Role of DS in IL-12-induced IFN-γ Secretion

Chapter 4 of this thesis focused on the functional role of GAGs/PGs, particularly the role of DS in NK cell regulation. The current study presented data that confirmed the previously demonstrated role of NK cell-surface DS in the regulation of innate IFN-γ production in response to IL-12 using chondroitinase ABC and β-xyloside treatment (Garnier *Et. al.*, 2003). In addition, the current study further extended the previous findings (Garnier *Et. al.*, 2003) by the step-wise investigation of the underlying

mechanism by which DS-PG mediates IL-12-signalled IFN-γ secretion. Firstly, the results indicated no significant effect of DS-PG on phosphorylation of the IL-12-specific transcription factor, STAT-4. Secondly, the data that the IFN-γ protein was not sequestered intracellularly eliminated the possibility that DS-PG affects the translational or post-translational events in IFN-γ expression. Therefore, this study concludes that DS-PG potentially modulates IL-12 activity in IFN-γ expression at the transcriptional stage. This is well-supported by a recent finding which demonstrated low levels of IFN-γ in decorin, a DS-PG KO mouse, leading to a reduced allergy-induced inflammatory response. Although the absence of DS-PG resulted in reduced IFN-γ mRNA levels and hence reduced protein expression in tissues of decorin KO mice, the mechanism of this transcriptional regulation of IFN-γ gene by DS-PG is still unknown (Bocian *Et. al.*, 2013).

As discussed earlier in Section 4.3, that one potential mechanism in the transcriptional control of IFN-γ expression by DS-PG could be p38 MAPK signalling. This signalling pathway has been indicated in both the direct and indirect regulation of IL-12 dependent IFN-γ expression (Visconti *Et. al.*, 2000; Zhang & Kaplan, 2000; Watford *Et. al.*, 2004). Hence, it would be important to determine whether DS-PG affects IFN-γ expression via p38 MAPK signalling. This can be experimentally demonstrated by stimulation of β-xyloside or chondroitinase ABC treated NK cells with IL-12 in the presence of the p38 inhibitor, SB203580. In this context, the effect of DS on p38 MAPK signalling pathway has been reported. For example, DS was shown to regulate osteoclast formation, in part, by inhibiting phosphorylation of p38 MAPK and ERK in RANKL stimulated monocytic cells (Shinmyouzu *Et. al.*, 2007).

Also, as mentioned earlier and discussed in detail in Chapter 4 (Section 4.3), a limitation to the approach used in the current investigation was encountered. This was due to possible cellular effects in addition to altered PG synthesis on NK cells by *p*-nitrophenyl-β-xyloside . Thus, it was not possible to continue and test the above proposed hypothesis that the functional role of DS-PG in IL-12-mediated IFN-γ transcription and production is mediated by p38 MAPK pathway. Since our work, in addressing this limitation, the recent advancements in this field of study have led to generation of new series of β-xylosides with a wide variety of aglycone structures and are known to be selective in inhibiting PG synthesis (Kuberan *Et. al.*, 2008; Tran *Et. al.*, 2013). These modified xylosides do not affect biosynthesis of other

glycoconjugates, like glycoproteins/glycolipids (Garud *Et. al.*, 2008). Some of these new xylosides are reported to prime not only synthesis of specific GAG chains, but also affect the pattern or extent of sulphation on those GAG chains (Victor *Et. al.*, 2009; Tran & Kuberan, 2014). Therefore, with more stability and versatility offered by these xylosides, they have been used in animal models, such as zebrafish, to understand the role of HS-PGs in developmental signalling events (Nguyen *Et. al.*, 2009). For example, these types of xylosides were successfully used by injecting in zebrafish embryos to demonstrate the effect of specific GAGs chains and valency, *in vivo*. Multimeric HS-PG primed on these added xylosides were shown to positively modulate FGF-FGFR signalling which resulted in an elongated phenotype during embryonic development (Nguyen *Et. al.*, 2013). Thus, one possibility to advance the current work would be the use of this new generation of xylosides.

Additionally, the novel phage display-derived antibodies, GD3A12 and LKN1 which can specifically recognise DS (den Dekker *Et. al.*, 2008; Ten Dam *Et. al.*, 2009) are now available. These antibodies can determine whether the β-xyloside/chondrotinase ABC treatment has resulted in significant, or complete removal of cell-surface DS-GAG chains. Moreover, these antibodies do not react with other classes of GAGs including CS and HS (Lensen *Et. al.*, 2006). Therefore, after the treatment with PG inhibitor or enzymes, decreased binding of these antibodies to the sulphated, cell-surface DS of NK cells could then be strongly correlated to IFN-γ production.

Broadly, the current findings also open more avenues to explore in this field that would highlight the importance of DS-GAGs/PGs in NK cell activation and effector functions. For instance, it remains to be determined whether NK cell-surface DS is also involved in regulating IL-12 induced activities other than IFN-γ production. IL-12 activated NK cells also produce other cytokines, such as TNF-α and GM-CSF, which contribute to innate immunity (Bellone *Et. al.*, 1995). Therefore, it would be a significant finding to determine whether the role of DS-PG is confined only to IFN-γ expression or could be extended to other NK cell-derived cytokines. In addition, DS-PG may have influence on other effector functions of NK cells. Regarding the tumour microenvironment, IL-12 enhanced NK cell cytolytic activity is one of the most important function. Whilst, it has been known that NK cell-CSPGs are mediators of target cell-lysis (Schmidt *Et. al.*, 1985), work with *p*-nitrophenyl-β-D-xyloside treated NK cells has been reported to possess unaltered cytolytic activity of these cells (Christmas *Et. al.*, 1988). Moreover,

exogenously added GAGs, CS-A, CS-C and DS showed no effect on NK cell-mediated cytotoxic activity (Yamamoto *Et. al.*, 1985). However, it is still not known if this cytolytic capacity of β-xyloside cultured NK cells remains unaffected upon IL-12 stimulation. This could be demonstrated by cytotoxicity assays using NK cells and target cell lines, YAC-1 (Karlhofer *Et. al.*, 1995) or K562 (Bellone *Et. al.*, 1995) in the presence of IL-12 and β-xyloside. This would indicate whether altered GAG/PG expression plays a role in modulating IL-12 activity in mediating NK cell functions and in doing so influences development of Th1 immune responses.

Since this investigation commenced, there have been no studies reporting the mechanistic role of NK cell-surface DS-PG in IL-12 induced IFN-γ expression that is important in understanding the influence of GAGs/PGs in cytokine expression and thereby contribution to immune responses. In this context, while DS-PG was shown as a key PG in the endometrial localization of CD16 $^{(-)}$ NK cells from the peripheral circulation (Kitaya & Yasuo, 2009), recent findings by Seidler et al demonstrated the role of decorin, a DS-PG, in the network of signalling pathways regulating cytokine expression, *in vivo* and *in vitro* (Bocian *Et. al.*, 2013). Consistent with our finding, the absence of DS-PG resulted in transcriptionally reduced IFN-γ expression in endothelial cells, thereby influencing reduced recruitment of $CD8^+$ T cells to inflamed tissue. Further, using $DCN^{-/-}$ mice, loss of decorin was shown to affect IFN-γ-induced STAT-1 signalling in chemokine CXCL-10 expression, thus explaining molecular basis to the role of DS-GAG/PG in modulating DTH (delayed type hypersensitivity), an allergic inflammatory response (Seidler *Et. al.*, 2011; Bocian *Et. al.*, 2013).

Moreover, unlike the current study, alternative studies conducted in this field have demonstrated the effect of exogenously added GAGs on cytokine expression. Some of these studies have also attempted to elucidate the underlying signalling mechanism. Yet, the study of the precise mechanisms is at an early stage and as the field develops, it will be interesting to learn from more examples. For instance, immuno-modulatory effects of soluble CS-A and CS-C were studied in LPS-stimulated TLR-4 pathway in chondrocytes. This was shown to be mediated by inhibition of a transcriptional regulator, NF-κB which consequently reduced inflammation (Campo *Et. al.*, 2009). Similarly, expression of several pro-inflammatory cytokines (IL-6, TNF-α) was shown to be strongly inhibited by CS-C in part, through suppression of NF-κB activation in LPS-stimulated macrophages (Tan & Tabata, 2014). However, the mechanism through

which CS-C inhibits NF-κB activation is still unknown. Hence, the current study and those described above exhibit continued efforts being directed towards understanding how signalling mechanisms are initiated and regulated by GAG/PGs, particularly in cytokine expression. This could be helpful in the design of effective immunomodulatory therapies for inflammation-associated diseases such as autoimmunity.

6.3 Role of TGF-β1 in IL-12-induced IFN-γ Secretion

The current study (Chapter 5) provides a molecular explanation for how TGF-β1 might regulate early IFN-γ production in NK cells in the presence of IL-12. On TGF-β1 treatment, IL-12 induced IFN-γ production was significantly inhibited in NK cells. Although this observation is consistent with number of earlier studies (Bellone *Et. al.*, 1995; Bright & Sriram, 1998; Sudarshan *Et. al.*, 1999; Laouar *Et. al.*, 2005; Lindgren *Et. al.*, 2011; Lewis *Et. al.*, 2015), an important aspect of the current research was to study this inhibitory effect of TGF-β1 independent of NK cell proliferation. This was supported by the early kinetics data which clearly demonstrated TGF-β1 suppressed IFN-γ production within 3-6 hours. This suggests that the inhibitory effect by TGF-β1 on NK cells preceded its action on cell transit through cell cycle. Such TGF-β1 dependent down-regulation of early IFN-γ production is likely to be significant *in vivo*, as NK cell-derived IFN-γ is an important component of tumour surveillance (Fauriat *Et. al.*, 2010; Wilson *Et. al.*, 2011) and has been implicated in Ag-independent and innate protection against various infections by microorganisms (Lewis *Et. al.*, 2015).

To investigate the interference of TGF-β1 in IL-12 signalled IFN-γ production, a systematic approach was adopted that tested the key components of the IL-12 signal transduction pathway in IFN-γ expression. Results on this clearly showed that IFN-γ inhibition by TGF-β1 was not dependent on IL-12-activated STAT-4 via the JAK-STAT pathway. In addition, the results also showed that TGF-β1-mediated early IFN-γ inhibition was not directly mediated by downregulation of a positive *trans*-activator of the IFN-γ gene, T-bet expression (Szabo *Et. al.*, 2000; J. Y. Cho *Et. al.*, 2003). Since transcriptional regulation is a complex process that requires several transcription factors in a collaborative effort controlling promoter-driven transcription, TGF-β1 might inhibit binding of T-bet and other significant factors that may co-operatively function in *trans*-activation of the IFN-γ promoter (Soutto *Et. al.*, 2002; J. Y. Cho *Et.*

al., 2003). To test this hypothesis, reporter assays were conducted using a -777bp to +48bp region of the proximal IFN-γ promoter that contained interaction sites for T-bet and notably, for various other transcription factors. These assays demonstrated that IL-12 activation of the IFN-γ promoter construct was insensitive to TGF-β1-induced repression suggesting TGF-β1 neither targets the direct action of T-bet in binding to its functional sites, nor inhibits the indirect role of T-bet influencing other factors in IFN-γ promoter activation. Importantly, results from reporter studies revealed that the TGF-β1 sensitive regulatory region could be further upstream of -777bp from the TSS of the IFN-γ gene. However, as mentioned earlier,this work on promoter analysis is preliminary and should be substantiated with more promoter mapping or deletion studies in future. This will enable the regions of the IFN-γ promoter that may be both necessary and sufficient for TGF-β1-induced IFN-γ repression to be determined. In conclusion, this study on signaling cross-talk between TGF-β1 and IL-12 has demonstrated that molecular regulation of early IFN-γ expression is independent of conventional and direct role of two key transcriptional factors, STAT-4 or T-bet in NK cells. Indeed, as discussed and proposed earlier in Chapter 5 (Section 5.3), the current data on early and rapid inhibition of IFN-γ production by TGF-β1, rather, highlights a possibility to explore and evaluate that TGF-β1 mediates repression of the *Ifng* locus through chromatin remodeling events such as transcriptionally repressive histone modifications which silences early *Ifng* expression.

Knowing the significance of IFN-γ in immune processes, it is not surprising that the regulation of this cytokine expression is complex and has been suggested to act at many different levels such as epigenetic, transcriptional and post-transcriptional (Schoenborn & Wilson, 2007; Young & Bream, 2007; Mah & Cooper, 2016; Leong *Et. al.*, 2017). Besides epigenetic modifications such as chromatin remodeling of the *Ifng* locus by TGF-β1, from the current findings, transcriptional and post-transcriptional events are other possible avenues to explore. This will provide a more detailed view of IFN-γ regulation by TGF-β1 in NK cells. As explained earlier, NK cells exhibit a constitutively 'open' status of the *Ifng* locus which promotes accessibility to transcription factors (Tato *Et. al.*, 2004). This open configuration of the *Ifng* locus has also been shown to correlate with the presence of constitutive IFN-γ transcripts in these cells and their ability to rapidly produce IFN-γ protein on activation (Stetson *Et. al.*, 2003). Further, stimulation with cytokines IL-2/IL-12 has shown to increase the stability of IFN-γ transcripts, nuclear transport and processing of these transcripts in

NK cells (Hodge *Et. al.*, 2002). Thus, NK cells are poised with constitutive IFN-γ mRNA expression which are further induced on cytokine stimulation. However, very low levels of IFN-γ protein have been suggested in the translation of this basal gene transcription (Stetson *Et. al.*, 2003; Tato *Et. al.*, 2004). Therefore, this leads to two possibilities in the control of NK cell production of IFN-γ by TGF-β1. First, TGF-β1 may directly act on IFN-γ transcription and secondly, TGF-β1 may affect a post-transcriptional mechanism such as mRNA stability.

Supporting the first hypothesis of transcriptional regulation by TGF-β1, earlier studies have shown that addition of TGF-β1 to NK cells suppressed IFN-γ mRNA levels concurrent with IFN-γ protein levels (Sudarshan *Et. al.*, 1999; Hayashi *Et. al.*, 2003; Laouar *Et. al.*, 2005). Since the current data demonstrates TGF-β1 mediated early and rapid inhibition of NK cell produced IFN-γ (3-6hrs), it therefore becomes an imminent need to measure IFN-γ mRNA levels by RT-PCR as part of future work. This will be insightful to determine the transcriptional control by TGF-β1. While, in support of the second hypothesis, IL-18 induced IFN-γ transcriptional activity in NK cells was shown to be unaffected by TGF-β1. Instead, it was shown to decrease the stability of IFN-γ mRNA leading to degradation of transcripts and hence reduced protein levels (Hayashi *Et. al.*, 2003). Hence, it will also be important to consider the latter possibility of post-transcriptional regulation of IFN-γ to further explore the findings from the current study. This will enable to determine whether TGF-β affects post-transcriptional events, such as IFN-γ mRNA stability. Rapid decay of IFN-γ mRNA could be a potential molecular mechanism in 'switching off' the IFN-γ expression by TGF-β1.

mRNA degradation is central to the post-transcriptional regulation and controlled by AU-rich elements (AREs), which are typically present as a pentameric sequence of AUUUA in the 3' untranslated region (3'UTR) of mRNAs (C. Y. Chen & Shyu, 1995). IFN-γ mRNA is one such labile transcript that contains several typical AU-rich elements (AREs) present in the 3' untranslated region (3'UTR) (Nagy *Et. al.*, 1994; Ye *Et. al.*, 1995). Investigations at this level of IFN-γ regulation is gaining increasing focus in recent years (Schwerk & Savan, 2015; Leong *Et. al.*, 2017). The importance of this region was shown by targeted substitution of a conserved 162 AU-rich nucleotide sequence in the 3'UTR region of IFN-γ with random nucleotides in a mouse model. This resulted in low, chronic IFN-γ mRNA and protein expression in the circulation indicating disrupted post-transcriptional control of IFN-γ gene (Hodge *Et. al.*, 2014).

However, little is known about the *trans*-acting factors that are involved in or regulate ARE-mediated mRNA destabilization, though there are proteins known to bind ARE sequences that participate in mRNA degradation. For example, earlier studies reported, HuR, as an ARE binding protein that regulated mRNA stability (Peng *Et. al.*, 1998). In recent years, TTP (Tristetraprolin), an ARE-binding protein was shown to mediate degradation of IFN-γ transcripts in T lymphocytes by its direct interaction with AUUUA-rich motifs on IFN-γ mRNA. Moreover, TTP deficient T cells showed that absence of this protein resulted in an increased half-life of IFN-γ mRNA and increased expression on activation (Ogilvie *Et. al.*, 2009). Since the current study demonstrated rapid inhibition of IFN-γ protein production by TGF-β1, it forms an attractive hypothesis that TGF-β1 mediates its effect on IFN-γ mRNA stability. Thus, TGF-β1 may alter the stability of IFN-γ mRNA induced by IL-12 alone or in synergy with IL-18 and may result in rapid decay of these transcripts. To address this mechanism, NK cells treated with or without TGF-β1 should be stimulated with IL-12 alone or IL-12 plus IL-18 in the presence and absence of a transcriptional inhibitor, actinomycin D. Quantifying IFN-γ mRNA at different time-points over the treatment with actinomycin D would enable detection of IFN-γ mRNA decay. Therefore, comparing the half-life of IFN-γ mRNA in IL-12 stimulated NK cells in the absence and presence of TGF-β1 would further suggest whether IFN-γ mRNA stability is affected by a TGF-β1-dependent signal. Further investigations will be required to elucidate the role of TGF-β1 in the induction of ARE-binding proteins and hence regulation of IFN-γ mRNA stability in NK cells.

Additionally, another molecular mechanism that has attracted recent recognition in the post-transcriptional control of IFN-γ expression by TGF-β1 is the induction of microRNAs (miRNA) (Hata & Chen, 2016; Mah & Cooper, 2016). miRNAs are endogenously produced small (~22 nucleotides) noncoding RNAs that post-transcriptionally regulate gene expression by inducing target mRNA degradation or inhibiting their translation (Bartel, 2009). They exert a regulatory role by binding to a partial complementary sequence in the 3' UTR region of a target mRNA (Schwerk & Savan, 2015). These miRNAs are emerging as critical players in the negative regulation of TGF-β-controlled gene expression and can regulate 100's of mRNAs simultaneously (Blahna & Hata, 2013; Hata & Chen, 2016). miRNAs are known to regulate IFN-γ production in NK and T cells (Savan, 2014; Leong *Et. al.*, 2017). An interesting study suggested a comprehensive role of miRNAs in IFN-γ production by demonstrating that

Dicer-deficient $CD4^+$ T cells which lack an important enzyme for miRNA processing, exhibited Th1 programming with secretion of high amounts of IFN-γ (Muljo *Et. al.*, 2005). Notably, miR-29, a specific miRNA expression has been shown to suppress IFN-γ production in NK and T cells by binding directly to the 3'UTR region of IFN-γ mRNA and inhibiting translation (F. Ma *Et. al.*, 2011). In contrast, another study demonstrated that miR-29 regulates IFN-γ production in T cells by directly targeting 3'UTR binding sites in degradation of T-bet and Eomes mRNAs, two key transcriptional regulators of IFN-γ expression (Steiner *Et. al.*, 2011). Very recently, up-regulated expression of miR-29 in NK cells from leukaemic patients was shown to correlate with reduced expression of T-bet and Eomes. This also resulted in overall downregulation of NK cell maturation and activity suggesting a mechanism in evasion of immunosurveillance (Mundy-Bosse *Et. al.*, 2016). Additionally, miR-155 was also shown to positively regulate expression of IFN-γ in NK cells on co-stimulation with IL-12 and IL-18 (Trotta *Et. al.*, 2012). Given the important clinical implications of miRNAs, especially miR-29 and miR-155 in multiple cancers and viral infections (Wang *Et. al.*, 2013; Leong *Et. al.*, 2017), further insights into the role of miRNAs in the regulation of IFN-γ is important. Future investigations can evaluate the expression of these miRNAs on TGF-β1 treatment of NK cells in the context of the post-transcriptional regulation of IFN-γ suppression.

On the whole, this part of the research in the thesis demonstrated a role for TGF-β1 in the suppression of innate IFN-γ synthesis by NK cells that impacts T cell activation towards Th1 and cell-mediated immune responses. This is relevant to various infectious diseases, such as leishmaniasis and *L. monocytogenes*, where a rapid innate response is vital to clearance of the infection versus persistence leading to chronic infection (recent review Sanjabi *Et. al.*, 2017). Similar pathological relevance is supported by studies demonstrating that viral specific proteins enhance TGF-β production and its cellular activity (Karimi-Googheri *Et. al.*, 2014). Consistently, *in vivo* studies have shown that TGF-β signalling in NK cells reduced IFN-γ expression, limiting their early anti-viral response and increased susceptibility to infections, particularly MCMV and LCMV (Lewis *Et. al.*, 2015). The clinical implications of current findings are not limited to the context of viral infections; they are significantly important in tumour settings as well. The current findings demonstrated that the suppressive effects of TGF-β1 on NK cell-produced IFN-γ is independent of its effect on cell proliferation and survival. This is consistent with other studies showing tumour-secreted TGF-β1 suppressed activation

of NK cells but did not affect their survival as against the anti-proliferative and apoptotic effect of TGF-β1 on T cells suggesting that strategies blocking TGF-β activity has the potential to retrieve NK cell functions in tumour microenvironments (Sanjabi *Et. al.*, 2009; Wilson *Et. al.*, 2011).

Besides secretion of TGF-β1, tumours escape recognition from cytotoxic T cells by several other mechanisms such as shedding of tumour-Ags and loss of MHC class I expression (Drake *Et. al.*, 2006). In-fact, the latter mechanism is associated with the activation of non-specific, NK cell-directed tumour cell killing and secretion of IFN-γ that further aides in tumour surveillance (Waldhauer & Steinle, 2008). Notably, in recent years, NK cells have gained attention as an important cellular tool in various tumour immunotherapies (Morvan & Lanier, 2016). In addition to the suppressive effects of tumour-derived TGF-β on NK cells, another major source of TGF-β are the regulatory T cells (Tregs) that is another route to evasion of tumour immune surveillance (Wing *Et. al.*, 2010; Sanjabi *Et. al.*, 2017). Tregs have been shown to inhibit IFN-γ secretion of NK cells (Pedroza-Pacheco, 2013). Therefore, to date, challenges remain in the successful use of these NK cells in cancer therapies. Downregulation of effector functions of immune cells by TGF-β1 in the tumour microenvironment has led investigators in the field to direct their efforts in therapeutically targeting TGF-β1 signalling (as reviewed in Tu *Et. al.*, 2014). NK cell-based immunotherapies with strategies that target TGF-β1 signalling are now attracting interest and particular focus is on engineering patient-derived NK cells for re-infusion of these *ex-vivo* activated NK cells which are capable of boosted IFN-γ production (Tarek & Lee, 2014; Granzin *Et. al.*, 2017). However, the blockade of TGF-β1 signalling by drugs or agents has posed a challenge that requires balancing the immunosuppressive effects of TGF-β1 with the beneficial role of TGF-β1 in immune homeostasis (Flavell *Et. al.*, 2010; Akhurst, 2017). These observations and findings necessitate a thorough understanding of the mechanisms by which TGF-β1 regulates IFN-γ production from NK cells. Studies, including ours, to some extent, contribute in the elucidation of this underlying mechanism that unleashes the potential of NK cells in generating more effective tumour immunotherapies and underscores the relevance of current work to the progress made in this field until now.

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